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FEE AUTHORIZATION FOR FILING PATENT APPLICATIONS

Attorney's Docket No. __BSA 01-02__

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s):   **Carl W. Anderson, et al.**

Title: **DNA-PK Assay**

.. ..

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

    Transmitted herewith is a patent application and the items as checked below for the above-identified case:

Twenty-Five (25) Sheets of ____ Formal __X__ Informal Drawings.

_____ Information Disclosure Statement Under 37 C.F.R. 1.56 and 1.97.

_____ Preliminary Amendment. Please enter changes before calculating filing fee.

__X__ The filing fee is calculated below.

CLAIMS AS FILED

Type Of Claim	Number Filed	Included In Basic Fee	Number Extra	Rate (Small Entity)	Total Fee
Total Claims	111 - 20	=	91	X $9 =	$ 819.00
Independent Claims	20 - 3	=	17	X $40 =	$ 680.00
		Basic Filing Fee (Small Entity)		=	$ 355.00
		Total Filing Fee		=	$1,854.00

__X__ The Commissioner is hereby authorized to charge the payment of the total filing fee for this patent to Deposit Account No. **02-3977**.

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: **Carl W. Anderson, et al.**

Attorney Docket No.: **BSA 01-02**

For: **DNA-PK ASSAY**

Assistant Commissioner for Patents
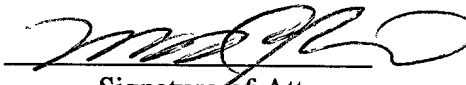Washington, D.C. 20231

Sir:

TRANSMITTAL

Applicants hereby submit the following documents:

a. The Original Patent Application identified above consisting of:
1. 143 page patent application
2. 111 claims
3. 25 sheets of informal drawings

b. Copy of unexecuted Combined Declaration and Power of Attorney

c. Transmittal Fee form authorizing payment to be charged to Deposit Account.

d. Small Entity form

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EXPRESS MAIL CERTIFICATE

Express Mail Label Number EL024708791US
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I hereby certify that the papers identified in this transmittal letter are being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Attention: Assistant Commissioner for Patents, Washington, D.C. 20231.

10/24/00
Date

Maria Pacella, Office of Economic Development and Technology Transfer

TITLE:

DNA-PK ASSAY

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The present application is a continuation-in-part of U.S. Patent Application

Serial No. 08/398,139 filed on March 3, 1995, which is a continuation-in-part of U.S.

Patent Application Serial No. 08/132,284 filed on October 6, 1993.

BACKGROUND OF THE INVENTION

1.   Field of the Invention

The present invention relates to compositions and methods useful for detecting

the presence and quantifying the amount of double-stranded DNA-activated protein

kinase (DNA-PK) in a biological sample and a method of monitoring DNA-PK activity

or DNA-PK activity state in living cells.

2.   Background of the Related Art

Eukaryotic cells contain many different protein kinases, each having specific

functions and properties. Protein kinases are important signal transduction enzymes

that regulate many aspects of cell metabolism and cell growth in eukaryotic cells.

Protein kinases alter the properties of other enzymes or structural proteins by

transferring a phosphate from a donor molecule, often ATP, to one or more acceptor

amino acids of a substrate protein. Although the basic amino acids lysine, arginine and

histidine can be phosphorylated by certain protein kinases which are primarily

associated with bacterial organisms, the most common and more widely recognized

phosphate acceptor amino acids in protein substrates in eukaryotic organisms are serine,

threonine and/or tyrosine residues. The most common and more well-studied protein

kinases are either protein-serine/threonine kinases, which are capable of transferring

phosphate groups from donor molecules to serine and threonine acceptor amino acid

residues in substrate proteins, or protein-tyrosine kinases, which are capable of

transferring phosphate groups from donor molecules to tyrosine acceptor amino acid

residues in substrate proteins.

Protein kinases have been found to modulate the activities of proteins in cells by

phosphorylating their specific protein substrates. These specific phosphorylation

reactions constitute a major mechanism for regulating biochemical pathways in

multicellular eukaryotic organisms. Nuclear events, metabolic processes and signal

transduction at the cytoplasmic membrane are coordinated through the phosphorylation

and dephosphorylation of proteins that perform and control these processes.

Transcription and DNA replication are also regulated by phosphorylation. Most

replication and transcription factors are phosphorylated at several sites and often by

several different protein kinases. Kinases have been described that are activated by a

variety of agents including cyclic nucleotides, phorbol esters, phospholipids, calcium

ions, heme groups, double-stranded RNA (dsRNA) and double-stranded DNA

(dsDNA). A growing number of nuclear regulatory proteins are known to be

phosphorylated, but the kinases which phosphorylate these proteins frequently remain

unidentified. Furthermore, many of the kinases which have been identified are not well characterized.

The characterization of many protein kinases has been facilitated by the development of *in vitro* protein kinase assays that specifically and quantitatively detect a particular kinase in a biological sample. Early work in this area was performed by Glass, et al. and is reported in *Anal. Biochem.*, 87:566 (1978). Glass, et al. developed a procedure for isolating phosphorylated peptides and proteins on ion exchange papers under acidic conditions that is applicable to the study of protein-serine/threonine and protein-tyrosine kinases. The method is not applicable to the study of protein kinases that phosphorylate the basic amino acids because the bond between the phosphate group and the basic amino acid is hydrolyzed in the acidic conditions of the method. The paper-binding method was applied to protein kinase assays that utilized synthetic peptides with amino acid sequences corresponding to the primary amino acid sequence at the phosphorylation sites of native proteins. Synthetic peptide substrates and methods for their rapid separation from kinase assay reaction mixtures have been useful in the detection, quantitation and characterization of numerous protein kinases.

Studies using substrate proteins and synthetic peptide substrates enabled the demonstration that a given protein kinase, whether of the protein-serine/threonine or the protein-tyrosine kinase class, did not phosphorylate all acceptor amino acid residues in a protein or peptide substrate, but was capable of specific selection of the serine, threonine or tyrosine that was to be phosphorylated in its various protein and peptide substrates. These studies lead to the determination that each protein kinase exhibited a specific phosphorylation site consensus sequence motif requirement for selection of the

acceptor amino acid (phosphorylation site) in protein substrates or synthetic peptide

substrates. Phosphorylation site consensus sequence motifs are comprised of a

phosphorylation site acceptor amino acid (serine, threonine or tyrosine) embedded in a

sequence or arrangement of amino acids that is specifically recognized by the protein

kinase such that the kinase transfers the phosphate to that acceptor amino acid rather

than to serines, threonines or tyrosines that are found elsewhere in the substrate but

which are not so embedded. Pearson, et al. in *Methods Enzymol.*, 220:62 (1991)

tabulated protein kinase phosphorylation site consensus sequence motifs for over 240

protein-serine/threonine and protein-tyrosine kinases. That tabulation was derived from

studies of protein kinases utilizing assays containing protein substrates or assays

containing synthetic peptide substrates that were synthesized as analogs of natural

phosphorylation site sequences.

Synthetic peptide analogs of phosphorylation site consensus sequence motifs are

useful for detecting, quantifying and characterizing protein kinases. Synthetic peptide

substrates have played an important role in the study of protein kinase (PK) substrate

specificity as well as in the measurement of protein kinase activities in cell extracts.

The major goals of designing synthetic peptide substrates for protein kinases are to

construct peptides that have excellent kinetic properties and a high degree of specificity

(Kemp, et al, 1991, *Methods Enzymol.*, 200:121; Pearson, et al., 1991, *Methods

Enzymol.*, 200:62).

While several synthetic peptides with amino acid sequences corresponding to the

primary amino acid sequence at the phosphorylated sites of proteins have been found to

serve as specific substrates for certain protein kinases, the degree of specificity of

synthetic peptide substrates varies widely. A number of synthetic peptides act as substrates for multiple protein kinases. For example, phosphorylase kinase, protein kinase C, and the multi-functional clad modulin-dependent protein kinase all phosphorylate Ser^7 in a glycogen synthase peptide.

Synthetic peptide substrates for protein kinases are particularly useful if they are specifically phosphorylated only by the protein kinase of interest. A specific synthetic peptide substrate comprises a kinase-specific phosphorylation site consensus sequence motif and sufficient additional amino acids (amino acid spacer sequences) so as to create a peptide providing excellent kinetic properties in an assay. Preferably, a specific synthetic peptide substrate provides a kinase-specific phosphorylation site consensus sequence motif and sufficient amino acid spacer sequences so as to provide excellent kinetic properties in an assay and which amino acid spacer sequences do not provide another phosphorylation site consensus sequence motif. Additionally preferable is a specific synthetic peptide substrate providing a kinase-specific phosphorylation site consensus sequence motif, sufficient amino acid spacer sequences to provide excellent kinetic properties in an assay that do not provide another phosphorylation site consensus sequence motif and which amino acid spacer sequences do not contain another phosphate acceptor amino acid. To provide for economic synthetic procedures, it is especially preferable that a specific synthetic peptide substrate be as short as possible while providing the features described above.

Recently, a kinase that undergoes activation by linear double stranded (ds) DNA was discovered. This kinase is now known as the double-stranded DNA-activated (or dependent) protein kinase (DNA-PK). DNA-PK is perhaps the most abundant nuclear

protein kinase in human cells. It is active *in vitro* only when certain double-stranded

DNA molecules are also present. Both natural DNAs and synthetic oligonucleotides

are DNA-PK activators. DNA-PK is a protein-serine/threonine kinase and therefore

phosphorylates certain serine and threonine residues in specific polypeptide sequences

by transferring a phosphate from the γ-position of a suitable phosphate donor, such as

adenosine triphosphate (ATP), to specific serine and threonine acceptor amino acids in

its substrate proteins.

DNA-PK phosphorylates several viral and cellular proteins *in vitro*, including

the simian virus 40 large tumor antigen (SV40 TAg), heat shock protein (hsp90), the

human and murine p53 tumor suppressor proteins, and several transcription factors

including Sp1, Oct-1, Fos, Jun and Serum Response Factor (SRF). Many of these

proteins are DNA-binding proteins that function in RNA transcription, DNA

replication, DNA recombination and DNA repair. The ability of DNA-PK to

phosphorylate these regulatory proteins provides a means for elucidating important

regulatory pathways. DNA-PK may be useful for studying the extent to which

phosphorylation regulates gene expression and cell growth. Additionally, DNA-PK

may be an important cellular enzyme in that it may play a role in transcription

regulation, DNA replication, and/or DNA repair. DNA-PK may be a regulatory kinase

which may be useful in detecting damaged DNA. The phosphorylation of cellular

protein substrates by DNA-PK may activate cell cycle checkpoint mechanisms that

arrest cell cycle progression in response to DNA damage. DNA-PK may also regulate

processes involved in the development of cellular immunity. These and other

properties suggest that DNA-PK may be extremely important for regulating the state or utilization of cellular DNA, especially in primate cells.

To determine the function of DNA-PK, it is necessary to understand the factors that result in its activation in cells. A significant technical difficulty in characterizing and studying DNA-PK has been the lack of a simple, quantitative, specific assay analogous to the synthetic peptide substrate assays, as mentioned above, which are available for other protein-serine/threonine kinases and protein-tyrosine kinases. The procedures for the synthesis of peptides for the study of cyclic nucleotide-dependent protein kinases may be applicable to the preparation of synthetic peptides which are specific for DNA-PK.

Previous research has shown that purified SV40 TAg and murine p53 protein were phosphorylated *in vitro* on serines by the human DNA-activated protein kinase, DNA-PK (Lees-Miller, et al., 1990, *Mol. Cell Biol.*, 10:6472-6481). More recently it was shown that the 14-residue synthetic peptide corresponding to TAg Thr[661] to Pro[674] was phosphorylated by DNA-PK primarily on the serine equivalent to TAg residue 665 (Chen, et al., 1991, *J. Virol.*, 10:5131-5140).

Several attempts have been made to provide a quantitative specific assay for DNA-PK. However, these attempts have failed. All known natural protein substrates for DNA-PK are also phosphorylated by other protein kinases. For example, casein which is a substrate of DNA-PK, is inexpensive and readily available. Unfortunately, casein is phosphorylated by several protein-serine/threonine kinases and therefore its phosphorylation, after incubation with a biological sample, for example, would not be a specific measure of DNA-PK activity in the biological sample. Hsp90 is a more

specific DNA-PK substrate, but it can also be phosphorylated by CKII (and perhaps other kinases). In addition, although hsp90 is abundant and easy to purify, its purification requires several days' work. Further, the quantitation of hsp90 phosphorylation is cumbersome and the kinetic constants for hsp90 phosphorylation are less than ideal. Consequently, simple cost-effective methods for accurately detecting the presence of DNA-PK are needed.

A further complication in characterizing and studying DNA-PK has been the lack of a meaningful correlation between the activity observed *in vitro* and DNA-PK activity *in vivo*. DNA-PK is activated *in vitro* by linear double-stranded DNA fragments, by nicked or gapped double-stranded DNAs or by DNAs with single-to-double strand transitions (bubbles, forks, hairpins, etc.) (Morozov, et al., 1994, *J. Biol. Chem.* 269:16684-16688). The preparation of cell extracts, for the determination of DNA-PK activity, inevitably introduces nicks and breaks into endogenous cellular DNA. DNA-PK or its Ku targeting/regulatory domain binds to the DNA at these nicks or breaks and becomes artificially activated. This artificial activation of DNA-PK upon cell disruption to form cell extracts makes it impossible to measure the status of intracellular DNA-PK activity using an *in vitro* cell extract assay.

An intracellular, *in vivo* method for measuring the intracellular DNA-PK activity and the activation status of DNA-PK within a cell would provide an alternative strategy. Such a strategy could include a determination of the *in vivo* phosphorylation state of known substrates. However, nearly all of the putative physiological substrates of DNA-PK are proteins that are present in cells at low abundance, making quantitation difficult. In addition, these proteins are also phosphorylated by other protein kinases.

Consequently, it has not been possible to ascertain the activity state of DNA-PK in living cells by examining the phosphorylation state of suspected substrates. Further, determining the activity of DNA-PK in cells or changes in its activity by examining the state of endogenous substrates is also complicated by the fact that determining the phosphorylation state of these proteins most often requires incubating cells with radioactive $^{32}PO_4$. The radiation emitted by this compound produces single-strand and double-strand breaks in the endogenous cellular DNA which alters the activity of DNA-PK in living cells. In fact, as previously mentioned, part of the *in vivo* function of DNA-PK may be to detect such DNA damage. It is known, for example, that radioactive nucleic acid precursors (e.g. [^{3}H]-thymidine) cause an accumulation of the tumor suppressor protein p53, a putative DNA-PK substrate (Dover, et al.,1994, *J. Cell Sci.* 107:1181-1184). Further, p53 accumulation in response to DNA breaks and other damage arrests cell cycle progression in the G_1 phase of the cell cycle (Nelson, et al., 1994, *Mol. Cell. Biol.* 14:1815-1823).

In light of the foregoing, it is a purpose of the present invention to provide a composition that comprises a synthetic peptide substrate which is a specific substrate for DNA-PK and which therefore can be used to specifically detect, quantitate and monitor DNA-PK activity.

Another purpose of the present invention is to provide specific quantitative methods of detecting the presence of DNA-PK activity in a biological sample.

Another purpose of the present invention is to provide methods of monitoring DNA-PK activity in living cells.

A further goal of the present invention is to provide a method of identifying agents which modulate DNA-PK activity *in vivo* and *in vitro*.

A further purpose of the present invention is to provide a kit for *in vitro* detection of the presence and relative amount of DNA-PK in a biological sample.

An additional purpose of the present invention is to provide a kit for monitoring the intracellular activity and activation status of DNA-PK.

SUMMARY OF THE INVENTION

These and other purposes and goals are accomplished by the present invention which provides a composition that comprises a synthetic peptide substrate that is a specific substrate for DNA-PK and which therefore is useful to specifically detect, quantitate and monitor DNA-PK activity. The synthetic peptide substrate of the present invention is a peptide substrate defined by the following features to provide specific recognition and phosphorylation by DNA-PK: (1) a phosphate-accepting amino acid pair which may include serine-glutamine (Ser-Gln) (SQ), threonine-glutamine (Thr-Gln) (TQ), glutamine-serine (Gln-Ser) (QS), or glutamine-threonine (Gln-Thr) (QT); (2) enhancer amino acids which may include glutamic acid or glutamine immediately adjacent at the amino- or carboxyl- side of the amino acid pair and forming an amino acid pair-enhancer unit; (3) a first spacer sequence at the amino terminus of the amino acid pair-enhancer unit; (4) a second spacer sequence at the carboxyl terminus of the amino acid pair-enhancer unit, which spacer sequences may include any combination of amino acids that does not provide a phosphorylation site consensus sequence motif; and, (5) a tag moiety, which may be an amino acid sequence or another chemical entity that permits separating the synthetic peptide from the phosphate donor.

Synthetic peptide substrates providing some or all of these features include: Met Glu Glu Pro Gln Ser Asp Pro Ser Val Glu Pro Pro Leu Ser Gln Glu Thr Phe Ser Asp Leu Trp Lys Leu Leu Pro Glu (SEQ ID NO: 1), Met Glu Glu Ser Gln Ser Asp Ile Ser Leu Glu Leu Pro Leu Ser Gln Glu Thr Phe Ser Gly Leu Trp Lys Leu Leu Pro Pro (SEQ ID NO: 2), Met Glu Glu Ser Gln Ser Asp Ile Ser Leu Glu Leu Pro Tyr Lys Lys (SEQ ID NO: 3), Met Glu Glu Pro Gln Ser Asp Pro Ser Val Glu Pro Pro Leu Ser Gln Glu Thr Phe Ser Asp Leu Trp Lys Lys (SEQ ID NO: 4), Asn Asn Val Leu Ser Pro Leu Pro Ser Gln Ala Met Asp Asp Leu Met Lys Lys (SEQ ID NO: 6), Met Ala Ile Tyr Lys Gln Ser Gln His Met Thr Glu Val Val Arg Arg (SEQ ID NO: 7), Glu Pro Pro Leu Ser Gln Glu Thr Phe Ser Asp Leu Trp Lys Lys (SEQ ID NO: 8), Glu Pro Pro Leu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 11), Glu Pro Pro Leu Ser Gln Glu Ala Phe Ala Asp Leu Leu Lys Lys (SEQ ID NO: 12), Glu Pro Pro Gln Ser Leu Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 14), Glu Pro Pro Gln Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 15), Glu Pro Pro Leu Thr Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 16), Glu Pro Pro Asp Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 17), Pro Glu Glu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 18) and Pro Glu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 19).

In a preferred embodiment the synthetic peptide substrate providing all of the features described above to provide specific recognition and phosphorylation by DNA-PK is a synthetic peptide substrate which is identical to or a variant of the amino acid sequence found at the amino terminus of human or murine p53 tumor suppressor proteins, and which contains either Ser[15] of human p53 tumor suppressor protein or Ser[7] or Ser[18] of murine p53 protein, including the following synthetic peptides: Met Glu Glu

Pro Gln Ser Asp Pro Ser Val Glu Pro Pro Leu Ser Gln Glu Thr Phe Ser Asp Leu Trp

Lys Leu Leu Pro Glu (SEQ ID NO: 1), Met Glu Glu Ser Gln Ser Asp Ile Ser Leu Glu

Leu Pro Leu Ser Gln Glu Thr Phe Ser Gly Leu Trp Lys Leu Leu Pro Pro (SEQ ID NO:

2), Met Glu Glu Ser Gln Ser Asp Ile Ser Leu Glu Leu Pro Tyr Lys Lys (SEQ ID NO:

3), Met Glu Glu Pro Gln Ser Asp Pro Ser Val Glu Pro Pro Leu Ser Gln Glu Thr Phe

Ser Asp Leu Trp Lys Lys (SEQ ID NO: 4), Glu Pro Pro Leu Ser Gln Glu Thr Phe Ser

Asp Leu Trp Lys Lys (SEQ ID NO: 8), Glu Pro Pro Leu Ser Gln Glu Ala Phe Ala Asp

Leu Trp Lys Lys (SEQ ID NO: 11), Glu Pro Pro Leu Ser Gln Glu Ala Phe Ala Asp

Leu Leu Lys Lys (SEQ ID NO: 12), Glu Pro Pro Gln Ser Gln Glu Ala Phe Ala Asp

Leu Trp Lys Lys (SEQ ID NO: 15), Glu Pro Pro Asp Ser Gln Glu Ala Phe Ala Asp

Leu Trp Lys Lys (SEQ ID NO: 17), Pro Glu Glu Ser Gln Glu Ala Phe Ala Asp Leu

Trp Lys Lys (SEQ ID NO: 18), Pro Glu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys

Lys (SEQ ID NO: 19), Pro Leu Ser Gln Glu Thr Phe Ser Gly Leu Trp Lys Leu Leu

Pro Pro-Lys Lys (SEQ ID NO: 63), and Pro Leu Ser Gln Glu Ala Phe Ala Gly Leu Trp

Lys Leu Leu Pro Pro-Lys Lys (SEQ ID NO: 64).

In a most preferred embodiment the synthetic peptide substrate providing all of

the features described above to provide specific recognition and phosphorylation by

DNA-PK is a synthetic peptide substrate which is a variant of the amino acid sequence

found at the amino terminus of human p53 tumor suppressor proteins, and which

contains Ser[15] of human p53 tumor suppressor protein, including the following peptides:

Glu Pro Pro Leu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 11),

Glu Pro Pro Gln Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 15),

Pro Glu Glu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 18) and Pro

Glu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 19).

The present invention also provides a quantitative method for detecting the

presence of DNA-PK activity in biological samples. The method includes forming a

reaction mixture by contacting a biological sample with a detectably-labeled phosphate

donor and a synthetic peptide substrate in the presence or absence of added exogenous

DNA. The synthetic peptide substrate is a peptide substrate defined by the following

features to provide specific recognition and phosphorylation by DNA-PK: (1) a

phosphate-accepting amino acid pair which may include serine-glutamine (Ser-Gln)

(SQ), threonine-glutamine (Thr-Gln) (TQ), glutamine-serine (Gln-Ser) (QS), or

glutamine-threonine (Gln-Thr) (QT); (2) enhancer amino acids which may include

glutamic acid or glutamine immediately adjacent at the amino- or carboxyl- side of the

amino acid pair and forming an amino acid pair-enhancer unit; (3) a first spacer sequence

at the amino terminus of the amino acid pair-enhancer unit; (4) a second spacer sequence

at the carboxyl terminus of the amino acid pair-enhancer unit, which spacer sequences

may include any combination of amino acids that does not provide a phosphorylation site

consensus sequence motif; and, (5) a tag moiety, which may be an amino acid sequence

or another chemical entity that permits separating the synthetic peptide from the

phosphate donor. Synthetic peptide substrates providing some or all of these features

include: Met Glu Glu Pro Gln Ser Asp Pro Ser Val Glu Pro Pro Leu Ser Gln Glu Thr Phe

Ser Asp Leu Trp Lys Leu Leu Pro Glu (SEQ ID NO: 1), Met Glu Glu Ser Gln Ser Asp Ile

Ser Leu Glu Leu Pro Leu Ser Gln Glu Thr Phe Ser Gly Leu Trp Lys Leu Leu Pro Pro

(SEQ ID NO: 2), Met Glu Glu Ser Gln Ser Asp Ile Ser Leu Glu Leu Pro Tyr Lys Lys

(SEQ ID NO: 3), Met Glu Glu Pro Gln Ser Asp Pro Ser Val Glu Pro Pro Leu Ser Gln Glu Thr Phe Ser Asp Leu Trp Lys Lys (SEQ ID NO: 4), Asn Asn Val Leu Ser Pro Leu Pro Ser Gln Ala Met Asp Asp Leu Met Lys Lys (SEQ ID NO: 6), Met Ala Ile Tyr Lys Gln Ser Gln His Met Thr Glu Val Val Arg Arg (SEQ ID NO: 7), Glu Pro Pro Leu Ser Gln Glu Thr Phe Ser Asp Leu Trp Lys Lys (SEQ ID NO: 8), Glu Pro Pro Leu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 11), Glu Pro Pro Leu Ser Gln Glu Ala Phe Ala Asp Leu Leu Lys Lys (SEQ ID NO: 12), Glu Pro Pro Gln Ser Leu Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 14), Glu Pro Pro Gln Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 15), Glu Pro Pro Leu Thr Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 16), Glu Pro Pro Asp Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 17), Pro Glu Glu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 18) and Pro Glu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 19).

In a preferred embodiment of this method, a biological sample is contacted with a synthetic peptide substrate which provides all of the features described above to provide specific recognition and phosphorylation by DNA-PK and which is identical to or a variant of the amino acid sequence found at the amino terminus of human or murine p53 tumor suppressor proteins, and which contains either Ser[15] of human p53 tumor suppressor protein or Ser[7] or Ser[18] of murine p53 protein, including the following synthetic peptides: Met Glu Glu Pro Gln Ser Asp Pro Ser Val Glu Pro Pro Leu Ser Gln Glu Thr Phe Ser Asp Leu Trp Lys Leu Leu Pro Glu (SEQ ID NO: 1), Met Glu Glu Ser Gln Ser Asp Ile Ser Leu Glu Leu Pro Leu Ser Gln Glu Thr Phe Ser Gly Leu Trp Lys Leu Leu Pro Pro (SEQ ID NO: 2), Met Glu Glu Ser Gln Ser Asp Ile Ser Leu

Glu Leu Pro Tyr Lys Lys (SEQ ID NO: 3), Met Glu Glu Pro Gln Ser Asp Pro Ser Val

Glu Pro Pro Leu Ser Gln Glu Thr Phe Ser Asp Leu Trp Lys Lys (SEQ ID NO: 4), Glu

Pro Pro Leu Ser Gln Glu Thr Phe Ser Asp Leu Trp Lys Lys (SEQ ID NO: 8), Glu Pro

Pro Leu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 11), Glu Pro

Pro Leu Ser Gln Glu Ala Phe Ala Asp Leu Leu Lys Lys (SEQ ID NO: 12), Glu Pro

Pro Gln Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 15), Glu Pro

Pro Asp Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 17), Pro Glu

Glu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 18), Pro Glu Ser Gln

Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 19), Pro Leu Ser Gln Glu Thr

Phe Ser Gly Leu Trp Lys Leu Leu Pro Pro-Lys Lys (SEQ ID NO: 63), and Pro Leu

Ser Gln Glu Ala Phe Ala Gly Leu Trp Lys Leu Leu Pro Pro-Lys Lys (SEQ ID NO:

64).

In a most preferred embodiment of this method, a biological sample is contacted

with a synthetic peptide substrate which provides all of the features described above to

provide specific recognition and phosphorylation by DNA-PK and which is a variant of

amino acid sequence found at the amino terminus of human p53 tumor suppressor

proteins, and which contains Ser[15] of human p53 tumor suppressor protein, including

the following peptides: Glu Pro Pro Leu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys

Lys (SEQ ID NO: 11), Glu Pro Pro Gln Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys

Lys (SEQ ID NO: 15), Pro Glu Glu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys

(SEQ ID NO: 18) and Pro Glu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ

ID NO: 19).

Next, the reaction mixture is incubated for a time at a temperature to allow the

transfer of phosphate from the phosphate donor to the acceptor amino acid in the

synthetic peptide substrate. After the incubation step, the transfer of phosphate from

the phosphate donor to the acceptor amino acid in the synthetic peptide substrate is

stopped. Finally, the method is used to determine the amount of phosphate which was

transferred from the phosphate donor to the acceptor amino acid in the synthetic peptide

substrate and to correlate the amount of phosphate transferred with a concentration of

DNA-PK in a biological sample.

In a preferred embodiment of the invention, the gamma phosphate of the

phosphate donor, ATP, contains a radioactive phosphorous isotope, e.g. [^{32}P] or [^{33}P].

After the incubation, the synthetic peptide, containing the radioactive phosphate

transferred by DNA-PK from the radiolabeled ATP, is separated from remaining

labeled ATP and phosphate using the tag moiety of the peptide substrate. In a preferred

embodiment, this separation is accomplished by absorbing the peptide to

phosphocellulose and washing the phosphocellulose with dilute acid to remove the

unreacted ATP phosphate donor. The amount of radioactivity transferred to the peptide

is then determined using a device, such as a scintillation counter, a beta particle

detector, gamma ray detector, or a phosphoimager, to quantitate the radioactivity in a

known portion of the reaction. The amount of phosphate transferred to the peptide is

then calculated from the known specific activity of the ATP.

A method of detecting the presence of linear double-stranded DNA in a

biological sample is also provided. The method includes forming a reaction mixture by

contacting a biological sample with a detectably-labeled phosphate donor, DNA-PK and

a synthetic peptide substrate defined by the following features to provide specific

recognition and phosphorylation by DNA-PK: (1) a phosphate-accepting amino acid

pair which may include serine-glutamine (Ser-Gln) (SQ), threonine-glutamine (Thr-Gln)

(TQ), glutamine-serine (Gln-Ser) (QS), or glutamine-threonine (Gln-Thr) (QT); (2)

enhancer amino acids which may include glutamic acid or glutamine immediately

adjacent at the amino- or carboxyl- side of the amino acid pair and forming an amino

acid pair-enhancer unit; (3) a first spacer sequence at the amino terminus of the amino

acid pair-enhancer unit; (4) a second spacer sequence at the carboxyl terminus of the

amino acid pair-enhancer unit, which spacer sequences may include any combination of

amino acids that does not provide a phosphorylation site consensus sequence motif; and

(5) a tag moiety, which may be an amino acid sequence or another chemical entity that

permits separating the synthetic peptide from the phosphate donor. The transfer of

phosphate from the phosphate donor to the peptide substrate is indicative of the

presence of linear double-stranded DNA in the sample because it is required for

activation of DNA-PK and was not supplied in the reaction mixture.

A method of detecting the presence of substances in a sample that alter the

activity of DNA-PK and DNA is also provided. The method includes forming a

reaction mixture by contacting increasing amounts of a sample with a detectably-labeled

phosphate donor, DNA-PK, linear double-stranded DNA and a synthetic peptide

substrate defined by the following features to provide specific recognition and

phosphorylation by DNA-PK: (1) a phosphate-accepting amino acid pair which may

include serine-glutamine (Ser-Gln) (SQ), threonine-glutamine (Thr-Gln) (TQ),

glutamine-serine (Gln-Ser) (QS), or glutamine-threonine (Gln-Thr) (QT); (2) enhancer

amino acids which may include glutamic acid or glutamine immediately adjacent at the amino- or carboxyl- side of the amino acid pair and forming an amino acid pair-enhancer unit; (3) a first spacer sequence at the amino terminus of the amino acid pair-enhancer unit; (4) a second spacer sequence at the carboxyl terminus of the amino acid pair-enhancer unit, which spacer sequences may include any combination of amino acids that does not provide a phosphorylation site consensus sequence motif; and, (5) a tag moiety, which may be an amino acid sequence or another chemical entity that permits separating the synthetic peptide from the phosphate donor. Changes in the amount or rate of transfer of phosphate from the phosphate donor to the peptide substrate caused by contacting the sample is related to the presence of substances that alter the activity of DNA-PK.

The present invention also provides a method of detecting the presence of protein phosphatases in a biological sample. The method includes contacting a biological sample with a phosphorylated synthetic peptide substrate defined by the following features to provide specific recognition by DNA-PK: (1) a phosphorylated amino acid pair which may include phosphoserine-glutamine (PO_4•Ser-Gln), phosphothreonine-glutamine (PO_4•Thr-Gln), glutamine-phosphoserine (Gln-PO_4•Ser), or glutamine-phosphothreonine (Gln-PO_4•Thr); (2) enhancer amino acids which may include glutamic acid or glutamine immediately adjacent at the amino- or carboxyl- side of the amino acid pair and forming an amino acid pair-enhancer unit; (3) a first spacer sequence at the amino terminus of the amino acid pair-enhancer unit; (4) a second spacer sequence at the carboxyl terminus of the amino acid pair-enhancer unit, which spacer sequences may include any combination of amino acids that does not provide a

phosphorylation site consensus sequence motif; and (5) a tag moiety, which may be an

amino acid sequence or another chemical entity that permits separating the synthetic

peptide from the phosphate donor. The loss of phosphate from the phosphorylated

peptide substrate is related to the presence of protein phosphatase in the sample.

The present invention also provides a composition useful in the specific,

quantitative detection of DNA-PK activity in a biological sample. The composition is a

synthetic peptide substrate defined by the following features to provide specific

recognition and phosphorylation by DNA-PK: (1) a phosphate-accepting amino acid

pair which may include serine-glutamine (Ser-Gln) (SQ), threonine-glutamine (Thr-Gln)

(TQ), glutamine-serine (Gln-Ser) (QS), or glutamine-threonine (Gln-Thr) (QT); (2)

enhancer amino acids which may include glutamic acid or glutamine immediately

adjacent at the amino- or carboxyl- side of the amino acid pair and forming an amino

acid pair-enhancer unit; (3) a first spacer sequence at the amino terminus of the amino

acid pair-enhancer unit; (4) a second spacer sequence at the carboxyl terminus of the

amino acid pair-enhancer unit, which spacer sequences may include any combination of

amino acids that does not provide a phosphorylation site consensus sequence motif; and

(5) a tag moiety, which may be an amino acid sequence or another chemical entity that

permits separating the synthetic peptide from the phosphate donor.

The present invention also provides a kit for detecting the presence of DNA-PK

20   activity in a biological sample for use in accordance with the method of the present

invention. The kit includes a detectably-labeled phosphate donor, double-stranded

DNA, a composition useful in specific detection and quantitation of DNA-PK which

comprises a synthetic peptide substrate that is phosphorylated by DNA-PK, a negative

control peptide that is not phosphorylated by DNA-PK, a preparation of DNA-PK and a reagent to separate the substrate peptide from the phosphate donor.

A negative control peptide that is not phosphorylated by DNA-PK may include a synthetic peptide with an amino acid sequence and composition similar to that of the synthetic peptide substrate, but with a variation of the phosphorylation site consensus sequence motif such that the acceptor amino acid is no longer phosphorylated by DNA-PK. Peptides with amino acid sequences and compositions that are similar to that of the synthetic peptide substrate may generally be comprised of the same amino acids as the peptide substrate with an alteration of the primary sequence such that the peptide no longer contains the appropriate phosphorylation site consensus motif and is thus not phosphorylated by the DNA-PK. Such negative control peptides thus provide non-phosphorylatable peptides that have the same ionic and physical properties as the phosphorylatable synthetic peptide substrate.

In the present invention, a negative control peptide may include a synthetic peptide identical in sequence to the synthetic peptide substrate with the single exception that the phosphorylatable serine or threonine of the amino acid pairs serine-glutamine (SQ), threonine-glutamine (TQ), glutamine-serine (QS), or glutamine-threonine (QT) of the synthetic peptide substrate is replaced with a non-phosphorylatable amino acid such as alanine or other neutral amino acid that does not alter the ionic character of the peptide.

In the present invention, a negative control peptide may also include a synthetic peptide identical in amino acid composition and sequence to the synthetic peptide substrate with the single exception that the enhancer amino acid glutamic acid is

inserted between the serine and glutamine of the serine-glutamine (SQ) amino acid pair,

or between the threonine and glutamine of the threonine-glutamine (TQ) amino acid, or

between the glutamine and the serine of the glutamine-serine (QS) pair or between the

glutamine and the threonine of the glutamine-threonine (QT) pair of the peptide rather

than being located immediately adjacent at the amino- or carboxyl-side of the amino

acid pair.

A method of monitoring intracellular protein kinase activity is also provided by

the present invention. The method includes introducing an expression vector into a

cell, in which the expression vector has the following features to provide monitoring

intracellular protein kinase activity: (1) a gene coding for a protein substrate having a

protein segment containing a phosphorylation site consensus sequence motif specific for

a protein kinase; (2) an optional nuclear localization signal; (3) an optional DNA-

binding domain; and (4) a detectable epitope. The expression of this protein substrate

provides a phosphorylatable protein substrate. By determining the amount of

phosphorylated protein substrate, a correlation to the concentration of protein kinase

activity in the cell is made.

The present invention also provides a method for monitoring intracellular DNA-

PK activity. The method includes introducing an expression vector into a cell, in which

the expression vector has the following features to provide for monitoring intracellular

DNA-PK activity: (1) a gene coding for a protein substrate having a protein segment

containing a phosphorylation site consensus sequence motif specific for DNA-PK; (2) a

nuclear localization signal; (3) a DNA-binding domain; and (4) a detectable epitope.

The expression of this protein substrate provides a phosphorylatable protein substrate.

By determining the amount of phosphorylated protein substrate, a correlation to the concentration of DNA-PK activity in the cell is made.

In either embodiment of the present invention for monitoring intracellular protein kinase activity, the protein substrate can further include: (1) an epitope for affinity purification; and (2) a cleavage site which permits excision of the phosphorylation site from the protein segment.

The present invention also provides a composition for monitoring intracellular protein kinase activity. The composition is an expression vector having the following features to facilitate monitoring protein kinase activity: (1) a gene coding for a protein substrate having a protein segment containing a phosphorylation site consensus sequence motif specific for a protein kinase; (2) an optional nuclear localization signal; (3) an optional DNA-binding domain; and (4) a detectable epitope.

A composition for monitoring intracellular DNA-PK activity is also provided by the present invention. The composition is an expression vector having the following features to facilitate monitoring DNA-PK activity: (1) a gene coding for a protein substrate having a protein segment containing a phosphorylation site consensus sequence motif specific for DNA-PK; (2) a nuclear localization signal; (3) a DNA-binding domain; and (4) a detectable epitope.

The present invention also provides a kit for monitoring intracellular protein kinase activity for use in accordance with the methods of the present invention. The kit includes: (1) an expression vector containing a gene coding for a protein substrate having a protein segment containing a phosphorylation site consensus sequence motif, in which the phosphorylation site consensus sequence motif is specific for a protein

kinase, a nuclear localization signal, a DNA-binding domain, and a detectable epitope; and (2) means for detecting a phosphorylated protein substrate. The detection of the phosphorylated protein substrate is utilized to determine the amount of protein kinase activity in the cell.

A kit for monitoring intracellular DNA-PK activity for use in accordance with the methods of the present invention is also provided. The kit includes: (1) an expression vector containing a gene coding for a protein substrate having a protein segment containing a phosphorylation site consensus sequence motif specific for DNA-PK, a nuclear localization signal, a DNA-binding domain, and a detectable epitope; and (2) means for detecting a phosphorylated protein substrate. The detection of the phosphorylated protein substrate is utilized to determine the amount of the DNA-PK activity in the cell.

The present invention also provides a method for identifying agents that alter the activity of a protein kinase in a cell. The method includes introducing an expression vector into a cell, in which the expression vector has the following features to facilitate identification of the agents: (1) a gene coding for a protein substrate having a protein segment containing a phosphorylation site consensus sequence motif specific for a protein kinase; (2) an optional nuclear localization signal; (3) an optional DNA-binding domain; and (4) a detectable epitope. The cell is contacted with the suspected agent.

20   The expression of the protein substrate by the cell provides a phosphorylatable protein substrate from which the amount of phosphorylated protein substrate is determined. The amount of phosphorylated protein substrate is then correlated to the concentration

of protein kinase activity in the cell and changes in the activity resulting from contacting the cell with the suspected agent.

A method for identifying agents that alter the activity of a DNA-PK in a cell is also provided by the present invention. The method includes introducing an expression vector into a cell, in which the expression vector has the following features to facilitate identification of the agents: (1) a gene coding for a protein substrate having a protein segment containing a phosphorylation site consensus sequence motif specific for DNA-PK; (2) a nuclear localization signal; (3) a DNA-binding domain; and (4) a detectable epitope. The cell is contacted with the suspected agent. The expression of the protein substrate by the cell provides a phosphorylatable protein substrate from which the amount of phosphorylated protein substrate is determined. The amount of phosphorylated protein substrate is correlated to the concentration of the DNA-PK activity in said cell and changes in the activity resulting from contacting the cell with the suspected agent.

The present invention also provides a method for monitoring a protein kinase activity in a cell through the expression of a reporter gene. The method includes introducing an expression vector into a cell, in which the expression vector has the following features to facilitate monitoring a protein kinase activity: (1) a gene coding for a protein substrate having a protein segment containing a phosphorylation site consensus sequence motif specific for the protein kinase; (2) an optional nuclear localization signal; (3) an optional DNA-binding domain; and (4) a detectable epitope. The expression of the protein substrate provides a phosphorylatable protein substrate, which is capable of activating expression of a reporter gene to provide a reporter gene

product. By determining the amount of reporter gene product expressed, a correlation

to the concentration of protein kinase activity in the cell is made.

A method for monitoring a DNA-PK activity in a cell through the expression of

a reporter gene is also provided by the present invention. The method includes

introducing an expression vector into a cell, in which the expression vector has the

following features to facilitate monitoring DNA-PK activity: (1) a gene coding for a

protein substrate having a protein segment containing a phosphorylation site consensus

sequence motif specific for DNA-PK; (2) a nuclear localization signal; (3) a DNA-

binding domain; and (4) a detectable epitope. The expression of the protein substrate

provides a phosphorylatable protein substrate, which is capable of activating expression

of a reporter gene to provide a reporter gene product. By determining the amount of

reporter gene product expressed, a correlation to the concentration of DNA-PK activity

in the cell is made.

For a better understanding of the present invention, reference is made to the

following description taken together with the accompanying drawings and sequence

listings, the scope of which is pointed out in the appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graphical representation of the rates of peptide phosphorylation

(nanomoles of phosphate incorporated per minute per milligram of DNA-PK protein) as

a function of peptide concentration for synthetic peptide (SEQ ID NO: 11) and synthetic

peptide (SEQ ID NO: 19). The amino acid sequences of (SEQ ID NOS: 11 and 19) are

listed in Table 1.

Figure 2 is a graphical representation of reciprocal plots used to determine the key substrate parameters, V_{max} and K_m, for reactions of DNA-PK with synthetic peptide (SEQ ID NO: 11) and synthetic peptide (SEQ ID NO: 18).

Figure 3 is a graphical representation of the rates of peptide phosphorylation (nanomoles of phosphate incorporated per minute per milligram of DNA-PK protein) as a function of peptide concentration for synthetic peptide (SEQ ID NO: 19), synthetic peptide (SEQ ID NO: 11), and synthetic peptide (SEQ ID NO: 18). The amino acid sequences of (SEQ ID NOS: 19, 11 and 18) are listed in Table 1.

Figure 4 is a schematic representation of six recombinant (artificial) DNA-PK substrates partially derived from human tumor suppressor gene product p53.

Figure 5A is a map of plasmid pT7HPOU1.

Figure 5B depicts the primary amino acid sequence of the expressed protein product of plasmid pT7HPOU1 (SEQ ID NO: 59).

Figure 5C depicts the nucleotide sequence for plasmid pT7HPOU1 (SEQ ID NO: 60).

Figure 6 is a map of plasmid pMAMneo.

Figure 6A is a map of plasmid pMACneo.

Figure 7 is a map of plasmid pMACSUB1.

Figure 8A is a map of plasmid p349SUB1.

Figure 8B depicts the primary amino acid sequence of the expressed product of plasmid p349SUB1 (SEQ ID NO: 61).

Figure 8C depicts the nucleotide sequence for plasmid p349SUB1 (SEQ ID NO: 62).

Figure 9 depicts the phosphorylation of recombinant protein substrates by purified

human DNA-PK as analyzed by SDS-PAGE. The upper portion is a phosphoimage of

the gel; lower portion is two panels showing Coosmassie blue stained images of regions

corresponding to hsp90 and the recombinant substrates respectively.

Figure 10 depicts a Coosmassie blue stained gel of the recombinant substrates of

the present invention analyzed by isoelectrofocusing gel electrophoresis.

Figure 11 is a map of plasmid p422SUB1 which encodes the DNA-PK wild-type

substrate (SEQ ID NO: 61).

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention a method and kit for detecting DNA-PK

activity are provided. The method includes forming a reaction mixture by contacting a

biological sample with a detectably-labeled phosphate donor and a synthetic peptide

substrate in the presence or absence of added exogenous DNA wherein the synthetic

peptide substrate is a peptide substrate defined by the following features to provide

specific recognition and phosphorylation by DNA-PK: (1) a phosphate-accepting amino

acid pair which may include serine-glutamine (Ser-Gln) (SQ), threonine-glutamine (Thr-

Gln) (TQ), glutamine-serine (Gln-Ser) (QS), or glutamine-threonine (Gln-Thr) (QT); (2)

enhancer amino acids which may include glutamic acid or glutamine immediately

adjacent at the amino- or carboxyl- side of the amino acid pair and forming an amino acid

pair-enhancer unit; (3) a first spacer sequence at the amino terminus of the amino acid

pair-enhancer unit; (4) a second spacer sequence at the carboxyl terminus of the amino

acid pair-enhancer unit, which spacer sequences may include any combination of amino

acids that does not provide a phosphorylation site consensus sequence motif; and (5) a tag

moiety, which may be an amino acid sequence or another chemical entity that permits

separating the synthetic peptide from the phosphate donor. Synthetic peptide substrates

providing some or all of these features include: Met Glu Glu Pro Gln Ser Asp Pro Ser

Val Glu Pro Pro Leu Ser Gln Glu Thr Phe Ser Asp Leu Trp Lys Leu Leu Pro Glu (SEQ

ID NO: 1), Met Glu Glu Ser Gln Ser Asp Ile Ser Leu Glu Leu Pro Leu Ser Gln Glu Thr

Phe Ser Gly Leu Trp Lys Leu Leu Pro Pro (SEQ ID NO: 2), Met Glu Glu Ser Gln Ser

Asp Ile Ser Leu Glu Leu Pro Tyr Lys Lys (SEQ ID NO: 3), Met Glu Glu Pro Gln Ser Asp

Pro Ser Val Glu Pro Pro Leu Ser Gln Glu Thr Phe Ser Asp Leu Trp Lys Lys (SEQ ID

NO: 4), Asn Asn Val Leu Ser Pro Leu Pro Ser Gln Ala Met Asp Asp Leu Met Lys Lys

(SEQ ID NO: 6), Met Ala Ile Tyr Lys Gln Ser Gln His Met Thr Glu Val Val Arg Arg

(SEQ ID NO: 7), Glu Pro Pro Leu Ser Gln Glu Thr Phe Ser Asp Leu Trp Lys Lys (SEQ

ID NO: 8), Glu Pro Pro Leu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID

NO: 11), Glu Pro Pro Leu Ser Gln Glu Ala Phe Ala Asp Leu Leu Lys Lys (SEQ ID NO:

12), Glu Pro Pro Gln Ser Leu Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 14),

Glu Pro Pro Gln Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 15), Glu

Pro Pro Leu Thr Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 16), Glu Pro

Pro Asp Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 17), Pro Glu Glu

Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 18) and Pro Glu Ser Gln

Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 19).

20   In a preferred embodiment of this method, a biological sample is contacted with

a synthetic peptide substrate which provides all of the features described above to

provide specific recognition and phosphorylation by DNA-PK and which is identical to

or a variant of the amino acid sequence found at the amino terminus of human or

murine p53 tumor suppressor proteins, and which contains either Ser[15] of human p53

tumor suppressor protein or Ser[7] or Ser[18] of murine p53 protein, including the following

synthetic peptides: Met Glu Glu Pro Gln Ser Asp Pro Ser Val Glu Pro Pro Leu Ser

Gln Glu Thr Phe Ser Asp Leu Trp Lys Leu Leu Pro Glu (SEQ ID NO: 1), Met Glu

Glu Ser Gln Ser Asp Ile Ser Leu Glu Leu Pro Leu Ser Gln Glu Thr Phe Ser Gly Leu

Trp Lys Leu Leu Pro Pro (SEQ ID NO: 2), Met Glu Glu Ser Gln Ser Asp Ile Ser Leu

Glu Leu Pro Tyr Lys Lys (SEQ ID NO: 3), Met Glu Glu Pro Gln Ser Asp Pro Ser Val

Glu Pro Pro Leu Ser Gln Glu Thr Phe Ser Asp Leu Trp Lys Lys (SEQ ID NO: 4), Glu

Pro Pro Leu Ser Gln Glu Thr Phe Ser Asp Leu Trp Lys Lys (SEQ ID NO: 8), Glu Pro

Pro Leu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 11), Glu Pro

Pro Leu Ser Gln Glu Ala Phe Ala Asp Leu Leu Lys Lys (SEQ ID NO: 12), Glu Pro

Pro Gln Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 15), Glu Pro

Pro Asp Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 17), Pro Glu

Glu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 18), Pro Glu Ser Gln

Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 19), Pro Leu Ser Gln Glu Thr

Phe Ser Gly Leu Trp Lys Leu Leu Pro Pro-Lys Lys (SEQ ID NO: 63), and Pro Leu

Ser Gln Glu Ala Phe Ala Gly Leu Trp Lys Leu Leu Pro Pro-Lys Lys (SEQ ID NO:

64).

In a most preferred embodiment of this method, a biological sample is contacted

with a synthetic peptide substrate which provides all of the features described above to

provide specific recognition and phosphorylation by DNA-PK and which is a variant of

amino acid sequence found at the amino terminus of human p53 tumor suppressor

proteins, and which contains Ser[15] of human p53 tumor suppressor protein, including

the following peptides: Glu Pro Pro Leu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys

Lys (SEQ ID NO: 11), Glu Pro Pro Gln Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys

Lys (SEQ ID NO: 15), Pro Glu Glu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys

(SEQ ID NO: 18) and Pro Glu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ

ID NO: 19).

Examples of amino acid tag moieties include but are not limited to (1) two or

more basic amino acids (arginine or lysine) that, in conjunction with the amino-

terminus, permit binding to phosphocellulose, (2) three or more acidic residues that

permit binding to an anion exchange resin, (3) six or more histidines that permit

binding to immobilized nickel or zinc ions and (4) an amino acid sequence that is

recognized by a monoclonal or polyclonal antibody.

Next, the reaction mixture is incubated, preferably at 30°C, for a time,

preferably for seven to ten minutes, facilitating the transfer of phosphate from the

phosphate donor to the synthetic peptide substrate. The transfer of phosphate to the

synthetic peptide substrate is stopped by the addition of an acid, preferably an equal

volume of 30% acetic acid, or by the addition of a sufficient amount of detergent or

other substance known to inhibit enzyme activity. The amount of labeled phosphate

transferred to the synthetic peptide is then determined. In a preferred method, to

determine the amount of labeled phosphate transferred to the synthetic peptide, the

reaction mixtures are spotted onto phosphocellulose filter paper and washed to remove

any unreacted labeled ATP phosphate donor molecules. The filter papers containing

the synthetic peptide are transferred to scintillation vials and the bound radioactivity is

determined from the Cerenkov radiation.

The amount of phosphate transferred to the synthetic peptide substrates is calculated and compared to negative controls which do not contain DNA-PK and positive controls which contain a known amount of DNA-PK. The amount of phosphate transferred to the synthetic peptide substrate is then correlated with a concentration of DNA-PK in the biological sample. In particular, the amount of phosphate transferred to the synthetic peptide substrate is calculated from the specific activity of the phosphate donor and that amount is compared to the amount of phosphate transferred in various negative control reactions including negative control reactions that do not contain synthetic peptide substrate, or those containing a negative control peptide that is not phosphorylated by DNA-PK, or those lacking activating DNA, or those which contain no biological sample or other source or preparation of DNA-PK. The amount of excess phosphate transferred to the substrate peptide compared to appropriate negative control reactions provides a specific measure of the amount of DNA-PK that is present. The assay may be used to compare the amounts of DNA-PK in two or more biological samples, or from the known specific activity of the kinase, to provide a minimal estimate of the absolute amount of active and/or activatable DNA-PK that is present in the sample.

All known natural protein substrates for DNA-PK are also phosphorylated by other protein kinases. Thus, phosphorylation of such natural protein substrates following contact with a biological sample is not completely attributable nor necessarily related to DNA-PK activity. An advantage of using the synthetic peptide substrate of the present invention which is specific for DNA-PK in the assay compared to using protein substrates known to be phosphorylated by DNA-PK (e.g. casein, hsp90, SV40

T-antigen), is that the measured activity is specific for DNA-PK because the other

protein kinases do not phosphorylate the synthetic peptide substrate of the present

invention.

Another advantage of using a synthetic peptide substrate of the present invention

which is specific for DNA-PK in the assay, compared to using protein substrates known

to be phosphorylated by DNA-PK (e.g. casein, hsp90, SV40 T-antigen), is that

appropriate negative control peptides may be used to ensure that the measured activity

is specific for DNA-PK. If a negative control peptide becomes phosphorylated

following contact with a biological sample it would suggest that some portion of the

activity measured with the DNA-PK peptide substrate may have been attributable to an

unknown protein kinase also contained in the biological sample. Thus, further testing

would be required to accurately assess the amount and activity of the DNA-PK in the

biological sample.

A negative control peptide that is not phosphorylated by DNA-PK may include a

synthetic peptide providing an amino acid sequence and composition similar to that of

the DNA-PK synthetic peptide substrate, but with a variation of the phosphorylation site

consensus sequence motif such that the acceptor amino acid is no longer recognized nor

phosphorylated by DNA-PK or other protein kinases. Peptides providing amino acid

sequences and compositions that are similar to those of the synthetic peptide substrate

may generally contain the same amino acids as the peptide substrate, but with an

alteration of the primary sequence such that the peptide no longer contains a

phosphorylation site consensus motif for DNA-PK and is thus not recognized nor

phosphorylated by the DNA-PK. Such negative control peptides thus provide non-

phosphorylatable peptides that have the same ionic and physical properties of the phosphorylatable synthetic peptide substrate.

In a preferred embodiment of the present invention, negative control peptides may include synthetic peptides identical in sequence to the synthetic peptide substrate with the single exception that the phosphorylatable serine or threonine of the amino acid pairs serine-glutamine (SQ), threonine-glutamine (TQ), glutamine-serine (QS), or glutamine-threonine (QT) of the synthetic peptide substrate is replaced with a non-phosphorylatable amino acid such as alanine or other neutral amino acid that does not alter the ionic character of the peptide.

In an additionally preferred embodiment of the present invention, negative control peptides may include synthetic peptides identical in amino acid composition and sequence to the synthetic peptide substrate with the single exception that the enhancer amino acid glutamic acid is inserted between the serine and glutamine of the serine-glutamine (SQ) amino acid pair, or between the threonine and glutamine of the threonine-glutamine (TQ) amino acid, or between the glutamine and the serine of the glutamine-serine (QS) pair or between the glutamine and the threonine of the glutamine-threonine (QT) pair of the peptide rather than being located immediately adjacent at the amino- or carboxyl-side of the phosphate-accepting amino acid pair.

Control peptides are used to estimate and correct for the incorporation of phosphate by DNA-PK into other peptides and proteins or other substances in the assay, or for the incorporation of phosphate by other kinases into assay components. As discussed, control peptides should not be phosphorylated by DNA-PK but, in general, should resemble substrate peptides in sequence and composition as closely as possible.

Thus, Glu Pro Pro Leu Ser Glu Gln Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO:

20) might serve as a negative control peptide for the substrate peptide Glu Pro Pro Leu

Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 11) since their

compositions are identical, but the negative control peptide (SEQ ID NO: 20) lacks the

mandatory phosphorylation site consensus sequence motif serine-glutamine (SQ) by

virtue of changing the Ser-Gln-Glu sequence of the substrate peptide to Ser-Glu-Gln in

(SEQ ID NO: 20). Similarly, Pro Glu Ser Glu Gln Ala Phe Ala Asp Leu Trp Lys Lys

(SEQ ID NO: 23) might serve as a negative control peptide for substrate peptide Pro

Glu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 19). (SEQ ID NO:

20) and (SEQ ID NO: 23) have the same compositions as substrate peptides (SEQ ID

NO: 11) and (SEQ ID NO: 19), respectively, but the order of the Gln and Glu are

reversed so as to disrupt the requisite serine-glutamine phosphate-accepting amino acid

pair of the DNA-PK synthetic peptide substrates and therefore they are not

phosphorylated by DNA-PK (Table 1). The peptide Glu Pro Pro Leu Ala Gln Glu Ala

Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 21) might serve as a negative control

peptide for the substrate peptide Glu Pro Pro Leu Ser Gln Glu Ala Phe Ala Asp Leu

Trp Lys Lys (SEQ ID NO: 11). Glu Pro Pro Leu Ala Gln Glu Ala Phe Ala Asp Leu

Trp Lys Lys (SEQ ID NO: 21) has an alanine substituted for the serine of the

phosphate accepting amino acid pair; and therefore has no site that can be

phosphorylated by a serine/threonine protein kinase but it is otherwise identical in

sequence to the substrate peptide. Similar to the above examples, negative control

peptides for the substrate peptide Pro Glu Glu Ser Gln Glu Ala Phe Ala Asp Leu Trp

Lys Lys (SEQ ID NO: 18) are represented by Pro Glu Glu Ala Gln Glu Ala Phe Ala

Asp Leu Trp Lys Lys (SEQ ID NO: 24) and Pro Glu Glu Ser Glu Gln Ala Phe Ala Asp

Leu Trp Lys Lys (SEQ ID NO: 25).

Glu Pro Pro Leu Ala Gln Glu Thr Phe Ser Asp Leu Trp Lys Lys (SEQ ID NO: 22) is an appropriate negative control peptide for Glu Pro Pro Leu Ser Gln Glu Thr Phe Ser Asp Leu Trp Lys Lys (SEQ ID NO: 8) since it does not have a Ser-Gln or Gln-Ser or Thr-Gln or Gln-Thr phosphate-accepting amino acid pair site for DNA-PK. SEQ ID NO: 22) is not phosphorylated efficiently by DNA-PK, but it does contain a serine and a threonine that might be recognized and phosphorylated by other kinases in biological samples. Thus, if the DNA-PK protein kinase activity of a biological sample was determined using (SEQ ID NO: 8) as the peptide substrate, using (SEQ ID NO: 22) as the negative control would appropriately test whether some of the phosphate incorporated following contact with the biological sample was attributable to the presence of an unknown protein kinase activity.

Conversely, peptides such as Pro Glu Glu Ala Gln Glu Thr Phe Ser Asp Leu Trp Lys Lys (SEQ ID NO: 26), for example, would be inappropriate negative control peptides for the substrate peptide Pro Glu Glu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 18) because of the presence of the serine and the threonine residues in (SEQ ID NO: 26) that might be recognized and phosphorylated by other kinases in biological samples and because of their absence from the peptide substrate (SEQ ID NO: 18).

In a particularly preferred embodiment, the gamma phosphate of the phosphate donor ATP, is labeled with a radioactive phosphorous isotope, e.g. [^{32}P] or [^{33}P]. After the incubation, the synthetic peptide, containing the radioactive phosphate transferred

by DNA-PK from the radiolabeled ATP, is separated from remaining labeled ATP and phosphate using the tag moiety. In one preferred embodiment, this separation is accomplished by absorbing the peptide to phosphocellulose and washing the phosphocellulose with dilute acid to remove unbound ATP. The amount of radioactivity transferred to the peptide is then determined using a device, such as a scintillation counter, a beta particle detector, gamma ray detector, or a phosphoimager, to quantitate the radioactivity in a known portion of the reaction. The amount of phosphate transferred to the peptide is then calculated from the known specific activity of the ATP. For example, if the specific activity of the ATP were 300 Ci/mole and the amount of radioactivity transferred to the peptide were 0.003 microcuries, then the amount of phosphate transferred to the peptide in the portion measured is 1 nanomole (0.003 μCi divided by 300 Ci/mole).

One unit of DNA-PK activity currently is defined as 1 nanomole of phosphate transferred per minute per mg of protein at 30°C at pH 7.5, 100 mM KCl, 25 mM HEPES buffer (standard assay conditions) with either hsp90 as a substrate or 200 μM synthetic peptide as a substrate as specified (Lees-Miller et al., 1990, *Mol. Cell. Biol.* 10: 6472-6481; Lees-Miller et al., 1992, *Mol. Cell. Biol.* 12:5041-5049). Using the peptide Glu Pro Pro Leu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 11) as the substrate, 1 mg of purified kinase provides approximately 50 units of DNA-PK activity under these conditions. This is a minimum estimate as the preparation is not completely pure and has not been optimized for the ratio of a 450-kDa catalytic subunit and a targeting subunit known as the Ku autoantigen. Both subunits are

required for activity with the peptide assay. Thus, a biological sample yielding a DNA-PK assay result of 1 unit of activity per milliliter would likely have a maximum DNA-PK concentration of approximately 20 µg/ml DNA-PK (1 U/ml divided by 50 U/mg).

In another preferred embodiment, exogenous linear double-stranded DNA is added to the reaction mixture as described in Example 4. The exogenous DNA may include DNA from the following list: plasmid DNA, viral DNA, bacteriophage DNA, calf thymus DNA, salmon testes DNA, salmon sperm DNA, herring sperm DNA, herring testes DNA, *Escherichia coli* DNA, *Micrococcus lysodeikticus* DNA, *Clostridium perfringens* DNA, *Clostridium welchii* DNA, human placenta DNA, synthetically prepared double-stranded DNA fragments, and sources of DNA including chromatin, viruses, cell nuclei and mixtures thereof. The synthetic double-stranded oligonucleotides may be $(dG-dC)_n$ and $(dC-dG)_n$, where n is equal to 7 to 15 (i.e. $(GCGC)_n$ or $(CGCG)_n$) and where the total length is 14 to 30 base pairs. Other synthetic oligonucleotides or DNAs that are not completely double stranded also may be used, for example, circular DNAs with gaps or bubbles, and linear DNAs with single stranded tails. It has been suggested that DNA-PK may have a physical requirement for a DNA end or free 5' nucleotide to become activated *in vitro* (Anderson and Lees-Miller, 1992, *Crit. Rev. in Eukaryotic Gene Expression*, 2:283-314). Therefore, the above list of DNAs is not exhaustive, but merely representative, as virtually any DNA can be manipulated to provide such an end or free 5' nucleotide and therefore function in the present invention.

The DNA is added to the reaction mixture to provide a concentration of DNA ranging from about 0.01 µg/ml to about 1000 µg/ml of the reaction mixture. In a

particularly preferred embodiment, the DNA is added to provide a concentration of from about 5 µg/ml to about 15 µg/ml of the reaction mixture, while in a most preferred embodiment the DNA is added to provide a concentration of DNA of about 10 µg/ml of the reaction mixture.

The detectably-labeled phosphate donor may be [^{32}P]-ATP, [^{32}P]-dATP, [^{33}P]-ATP, [^{33}P]-dATP and mixtures thereof. In a preferred embodiment the detectably-labeled phosphate donor is [^{32}P]-ATP. The [^{32}P]-ATP and non-radioactive ATP are added to the reaction mixture to provide a concentration of ATP ranging from about 20 µM to about 1 mM. The [^{32}P]-ATP has an approximate specific activity of 300mCi/mmole.

In principal, radioactive ATP is not required for detection of phosphate transferred to a peptide. Non-radioactive methods can be used to detect peptide substrate phosphorylation. Phosphorylation of the peptide can be determined from changes in absorbance, fluorescence or changes in the chemical or electrophoretic properties of the peptide. These methods may be coupled with methods for detecting phosphate or the peptide. For example, there are at least three methods for detecting the transfer of non-radioactive phosphate to a peptide: (1) increased mass of the peptide due to the addition of one or more phosphates; (2) increased negative charge of the peptide due to phosphate addition; and (3) binding of the peptide through the phosphate to chelated iron.

The masses of peptides are measured with sensitivity and accuracy using commercial mass spectrometers. A change in the charge of a peptide can be

determined by using thin-layer electrophoresis on cellulose plates. The peptide may be detected either by including a chromophor (e.g. nitro-phenylalanine, rhodamine coupled to lysine, etc.) in the peptide sequence, or by staining the peptide after electrophoresis with reagents specific for either the peptide sequence (e.g. a monoclonal antibody) or for a specific chemical property of the peptide (e.g. an amino group). Phosphate, including peptide-bound phosphate, binds very tightly to chelated iron at acid pH and is released at alkaline pH. Thus phosphopeptides can be separated from most non-phosphorylated peptides by passing the peptide mixture over a column of iron immobilized on Chelating-Sepharose (Sigma Chemical Co., St. Louis, MO) at acid pH and then eluting bound peptide with an alkaline buffer or phosphate. Increased sensitivity can be realized by combining two or more methods; e.g. iron-purified peptides could be subjected to mass analysis or separated by thin-layer electrophoresis. The amount of phosphate transferred to the peptide is quantitatively measured either from a direct chemical measurement of the phosphate in the purified phosphopeptide, or by quantitating the amount of phosphopeptide produced. This is determined by quantitative amino acid analysis of the purified phosphopeptide or by comparing it quantitatively to a phosphopeptide standard of known concentration.

In accordance with the method of the present invention, the synthetic peptide substrate is added to the reaction mixture to provide a concentration of substrate ranging from about 50 μM to about 1000 μM. In a preferred embodiment, the synthetic peptide substrate is added to provide a concentration of substrate ranging from about 100 μM to about 400 μM of the reaction mixture, while in a most preferred

embodiment synthetic peptide substrate is added to provide a concentration of substrate

of about 200 μM of the reaction mixture.

In carrying out the method, the reaction mixture is incubated from about 1

second to about 60 minutes. Preferably the reaction mixture is incubated from about 2

minutes to about 15 minutes, while in a most preferred embodiment the reaction

mixture is incubated from about 7 minutes to about 10 minutes. The reaction mixture

is incubated at a temperature ranging from about 5°C to about 45°C. In a preferred

embodiment the reaction mixture is incubated at a temperature from about 20°C to

about 37°C, while in a most preferred embodiment the reaction mixture is incubated at

a temperature of about 30°C.

The method of the present invention has other uses in addition to the detection

of DNA-PK activity in biological samples. For example, the method may be used to

detect double-stranded DNA in samples and to estimate the concentration of DNA in

such samples, to detect and estimate the concentration of a phosphate donor in samples,

to detect and study substances that alter any of these reagents, i.e. DNA-PK, DNA,

phosphate donor that would alter the activity of DNA-PK, and to detect and study

enzymes that remove phosphate from the substrate peptide.

In particular, by providing DNA-PK in a reaction, and omitting exogenous

DNA from the reaction, the method is configured to detect DNA in a biological

sample. The method includes forming a reaction mixture by contacting a biological

sample with a detectably-labeled phosphate donor, a source of DNA-PK and a synthetic

peptide substrate defined by the following features to provide specific recognition and

phosphorylation by DNA-PK: (1) a phosphate-accepting amino acid pair which may

include serine-glutamine (Ser-Gln) (SQ), threonine-glutamine (Thr-Gln) (TQ), glutamine-serine (Gln-Ser) (QS), or glutamine-threonine (Gln-Thr) (QT); (2) enhancer amino acids which may include glutamic acid or glutamine immediately adjacent at the amino- or carboxyl- side of the amino acid pair and forming an amino acid pair-enhancer unit; (3) a first spacer sequence at the amino terminus of the amino acid pair-enhancer unit; (4) a second spacer sequence at the carboxyl terminus of the amino acid pair-enhancer unit, which spacer sequences may include any combination of amino acids that does not provide a phosphorylation site consensus sequence motif; and (5) a tag moiety, which may be an amino acid sequence or another chemical entity that permits separating the synthetic peptide from the phosphate donor.

The method of detecting DNA in a biological sample is identical to the method of detecting DNA-PK, except that DNA-PK is added to the reaction mixture and exogenous DNA is not added. Since DNA-PK is dependent on the presence of linear double-stranded DNA or DNA with duplex-to-single-stranded transitions for catalyzing the transfer of phosphate, if such DNA is present in the biological sample the synthetic peptide substrate will be phosphorylated. The amount of phosphorylated substrate can then be correlated to a concentration of DNA in the biological sample. The amount of DNA in the sample would be estimated by comparing the amount of phosphorylated substrate obtained with dilutions of the sample to the amount of phosphorylated substrate obtained with known concentrations of DNA.

By including both a source or preparation of DNA-PK and linear double-stranded DNA in the reaction, the method is configured to detect substances that alter the activity of DNA-PK. The method includes forming a reaction mixture by

contacting a sample with a detectably-labeled phosphate donor, a source or preparation

of DNA-PK, a source or preparation of linear double-stranded DNA and a synthetic

peptide substrate defined by the following features to provide specific recognition and

phosphorylation by DNA-PK: (1) a phosphate-accepting amino acid pair which may

include serine-glutamine (Ser-Gln) (SQ), threonine-glutamine (Thr-Gln) (TQ),

glutamine-serine (Gln-Ser) (QS), or glutamine-threonine (Gln-Thr) (QT); (2) enhancer

amino acids which may include glutamic acid or glutamine immediately adjacent at the

amino- or carboxyl- side of the amino acid pair and forming an amino acid pair-

enhancer unit; (3) a first spacer sequence at the amino terminus of the amino acid pair-

enhancer unit; (4) a second spacer sequence at the carboxyl terminus of the amino acid

pair-enhancer unit, which spacer sequences may include any combination of amino

acids that does not provide a phosphorylation site consensus sequence motif; and (5) a

tag moiety, which may be an amino acid sequence or another chemical entity that

permits separating the synthetic peptide from the phosphate donor.

The method of detecting the presence of substances which alter the activity of

DNA-PK is identical to the method of detecting DNA-PK except that DNA-PK and

linear double-stranded DNA are both added to the reaction mixture. The presence of

DNA-PK activity altering substances in the sample can be determined by comparing the

amount of phosphorylated substrate obtained in the presence of sample contact to the

20   amount of phosphorylated substrate obtained in control reactions for which sample

contact was omitted. Similarly, this method will detect substances which alter the

DNA-PK activity by modifying the structure of the linear double-stranded DNA. The

result of such modification is detected by an increase or decrease in the catalytic

activity of DNA in the DNA-PK mediated phosphorylation reaction.

By prephosphorylating the substrate peptide with DNA-PK, the method is

configured to detect the presence in a biological sample of protein phosphatase that

removes phosphate from the substrate peptide. The method includes forming a reaction

mixture by contacting a biological sample with a phosphorylated synthetic peptide

substrate defined by the following features to provide specific recognition by DNA-PK:

(1) a phosphorylated amino acid pair which may include phosphoserine-glutamine

(PO_4•Ser-Gln), phosphothreonine-glutamine (PO_4•Thr-Gln), glutamine-phosphoserine

(Gln-PO_4•Ser), or glutamine-phosphothreonine (Gln-PO_4•Thr); (2) enhancer amino

acids which may include glutamic acid or glutamine immediately adjacent at the amino-

or carboxyl- side of the amino acid pair and forming an amino acid pair-enhancer unit;

(3) a first spacer sequence at the amino terminus of the amino acid pair-enhancer unit;

(4) a second spacer sequence at the carboxyl terminus of the amino acid pair-enhancer

unit, which spacer sequences may include any combination of amino acids that does not

provide a phosphorylation site consensus sequence motif; and (5) a tag moiety, which

may be an amino acid sequence or another chemical entity that permits separating the

synthetic peptide from the phosphate donor.

The method of detecting protein phosphatase in a biological sample uses

synthetic peptide substrates which have been prephosphorylated by DNA-PK. The

amount of phosphatase in the sample would be estimated by comparing the amount of

phosphorylated substrate remaining after the incubation period to the amount of

phosphorylated substrate remaining in control reactions.

A kit for detecting the presence of DNA-PK in a biological sample in accordance with the method of the present invention includes: (1) a detectably-labeled phosphate donor, (2) linear double-stranded DNA, (3) a composition useful in specific detection and quantitation of DNA-PK which comprises a substrate peptide that is phosphorylated by DNA-PK, (4) a negative control peptide of similar composition that is not phosphorylated by DNA-PK, (5) DNA-PK, and (6) a reagent to select or separate the substrate peptide.

Specific synthetic peptide substrates that are phosphorylated by DNA-PK may include: Met Glu Glu Pro Gln Ser Asp Pro Ser Val Glu Pro Pro Leu Ser Gln Glu Thr Phe Ser Asp Leu Trp Lys Leu Leu Pro Glu (SEQ ID NO: 1), Met Glu Glu Ser Gln Ser Asp Ile Ser Leu Glu Leu Pro Leu Ser Gln Glu Thr Phe Ser Gly Leu Trp Lys Leu Leu Pro Pro (SEQ ID NO: 2), Met Glu Glu Ser Gln Ser Asp Ile Ser Leu Glu Leu Pro Tyr Lys Lys (SEQ ID NO: 3), Met Glu Glu Pro Gln Ser Asp Pro Ser Val Glu Pro Pro Leu Ser Gln Glu Thr Phe Ser Asp Leu Trp Lys Lys (SEQ ID NO: 4), Asn Asn Val Leu Ser Pro Leu Pro Ser Gln Ala Met Asp Asp Leu Met Lys Lys (SEQ ID NO: 6), Met Ala Ile Tyr Lys Gln Ser Gln His Met Thr Glu Val Val Arg Arg (SEQ ID NO: 7), Glu Pro Pro Leu Ser Gln Glu Thr Phe Ser Asp Leu Trp Lys Lys (SEQ ID NO: 8), Glu Pro Pro Leu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 11), Glu Pro Pro Leu Ser Gln Glu Ala Phe Ala Asp Leu Leu Lys Lys (SEQ ID NO: 12), Glu Pro Pro Gln Ser Leu Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 14), Glu Pro Pro Gln Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 15), Glu Pro Pro Leu Thr Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 16), Glu Pro Pro Asp Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 17), Pro Glu Glu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys

Lys (SEQ ID NO: 18) and Pro Glu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ

ID NO: 19). The kit may be supplied with or without any or all of the above components,

and may also include buffers as appropriate for the intended user, except that it must

contain one or more substrate peptides.

In a preferred embodiment the synthetic peptide substrate providing all of the

features described above to provide specific recognition and phosphorylation by DNA-

PK is a synthetic peptide substrate which is identical to or a variant of the amino acid

sequence found at the amino terminus of human or murine p53 tumor suppressor

proteins, and which contains either Ser^{15} of human p53 tumor suppressor protein or Ser^7

or Ser^{18} of murine p53 protein, including the following synthetic peptides: Met Glu Glu

Pro Gln Ser Asp Pro Ser Val Glu Pro Pro Leu Ser Gln Glu Thr Phe Ser Asp Leu Trp

Lys Leu Leu Pro Glu (SEQ ID NO: 1), Met Glu Glu Ser Gln Ser Asp Ile Ser Leu Glu

Leu Pro Leu Ser Gln Glu Thr Phe Ser Gly Leu Trp Lys Leu Leu Pro Pro (SEQ ID NO:

2), Met Glu Glu Ser Gln Ser Asp Ile Ser Leu Glu Leu Pro Tyr Lys Lys (SEQ ID NO:

3), Met Glu Glu Pro Gln Ser Asp Pro Ser Val Glu Pro Pro Leu Ser Gln Glu Thr Phe

Ser Asp Leu Trp Lys Lys (SEQ ID NO: 4), Glu Pro Pro Leu Ser Gln Glu Thr Phe Ser

Asp Leu Trp Lys Lys (SEQ ID NO: 8), Glu Pro Pro Leu Ser Gln Glu Ala Phe Ala Asp

Leu Trp Lys Lys (SEQ ID NO: 11), Glu Pro Pro Leu Ser Gln Glu Ala Phe Ala Asp

Leu Leu Lys Lys (SEQ ID NO: 12), Glu Pro Pro Gln Ser Gln Glu Ala Phe Ala Asp

Leu Trp Lys Lys (SEQ ID NO: 15), Glu Pro Pro Asp Ser Gln Glu Ala Phe Ala Asp

Leu Trp Lys Lys (SEQ ID NO: 17), Pro Glu Glu Ser Gln Glu Ala Phe Ala Asp Leu

Trp Lys Lys (SEQ ID NO: 18), Pro Glu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys

Lys (SEQ ID NO: 19), Pro Leu Ser Gln Glu Thr Phe Ser Gly Leu Trp Lys Leu Leu

Pro Pro-Lys Lys (SEQ ID NO: 63), and Pro Leu Ser Gln Glu Ala Phe Ala Gly Leu Trp

Lys Leu Leu Pro Pro-Lys Lys (SEQ ID NO: 64).

In a most preferred embodiment the synthetic peptide substrate providing all of

the features described above to provide specific recognition and phosphorylation by

DNA-PK is a synthetic peptide substrate which is a variant of the amino acid sequence

found at the amino terminus of human p53 tumor suppressor proteins, and which

contains Ser^{15} of human p53 tumor suppressor protein, including the following peptides:

Glu Pro Pro Leu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 11),

Glu Pro Pro Gln Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 15),

Pro Glu Glu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 18) and Pro

Glu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 19).

In particular, in the preferred embodiment, the kit includes, calf thymus DNA

or the synthetic oligonucleotide $(dG-dC)_{12}$ (SEQ ID NO: 27), the substrate peptide

(SEQ ID NO: 11) or (SEQ ID NO: 18) or (SEQ ID NO: 19), the control peptide (SEQ

ID NO: 20) or (SEQ ID NO: 25) or (SEQ ID NO: 23), a concentrated HEPES buffer

solution, pH 7.5 containing 250 mM HEPES, pH 7.5, 1 M KCl, 100 mM $MgCl_2$, 1

mM EDTA, 1 mM EGTA, 10 mM DTT, phosphocellulose paper disks, and a set of

instructions.

When the above components of the kit are combined in the presence of DNA-

PK under the appropriate reaction conditions, the phosphate is transferred from the

detectably-labeled phosphate donor to the synthetic peptide substrate. Also included in

the kit may be a means for stopping the transfer of the phosphate to the synthetic

peptide substrate. Once the transfer of labeled phosphate is stopped, the amount of

phosphate transferred from the phosphate donor to the synthetic peptide substrate is detected. The amount of phosphate transferred to the synthetic peptide substrate is correlated to the concentration of DNA-PK in the biological sample as described in the method of the present invention.

The kit of the present invention has other uses in addition to the detection of DNA-PK activity in biological samples. For example, the kit may be used to detect double-stranded DNA in samples and to estimate the concentration of DNA in such samples, to detect and estimate the concentration of a phosphate donor in samples, to detect and study substances that alter any of these reagents, i.e. DNA-PK, DNA, phosphate donor that would alter the activity of DNA-PK, and to detect and study enzymes that remove phosphate from the substrate peptide. In particular, by including a source or preparation of DNA-PK and omitting a source or preparation of DNA in the reaction mixture, the kit may be used to detect DNA in a biological sample. The amount of DNA in the sample would be estimated by comparing the amount of phosphorylated substrate obtained with dilutions of the sample to the amount of phosphorylated substrate obtained with known concentrations of DNA. By including both a source or preparation of DNA-PK and DNA in a reaction mixture, the kit may be used to detect substances that alter the activity of DNA-PK. By prephosphorylating the substrate peptide with DNA-PK and including the phosphorylated peptide in a reaction mixture in the absence of a source or preparation of DNA-PK, the kit may be used to detect protein phosphatases that remove phosphate from the substrate peptide.

The linear double-stranded DNA utilized in the present invention may be DNA from the following list: plasmid DNA, viral DNA, bacteriophage DNA, calf thymus

DNA, salmon testes DNA, salmon sperm DNA, herring sperm DNA, herring testes

DNA, *Escherichia coli* DNA, *Micrococcus lysodeikticus* DNA, *Clostridium perfringens*

DNA, *Clostridium welchii* DNA, human placenta DNA, synthetically prepared double-

stranded DNA fragments, and sources of DNA including chromatin, viruses, cell nuclei

and mixtures thereof. The synthetic double-stranded oligonucleotides may be $(dG-dC)_n$

and $(dC-dG)_n$, where n is equal to 7 to 15 (i.e. $(GCGC)_n$ or $(CGCG)_n$) and where the

total length is 14 to 30 base pairs. Other synthetic oligonucleotides or DNAs that are

not completely double stranded also may be used, for example, circular DNAs with

gaps or bubbles, and linear DNAs with single stranded tails. It has been suggested that

DNA-PK may have a physical requirement for a DNA end or free 5' nucleotide to

become activated *in vitro* (Anderson and Lees-Miller, 1992, *Crit. Rev. in Eukaryotic*

Gene Expression, 2:283-314). Therefore, the above list of DNAs is not exhaustive, but

merely representative, as virtually any DNA can be manipulated to provide such an end

or free 5' nucleotide and therefore function in the present invention.

The detectably-labeled phosphate donor found in the kit of the present invention

may be $[^{32}P]$-ATP, $[^{32}P]$-dATP, $[^{33}P]$-ATP and $[^{33}P]$-dATP. In a particularly preferred

embodiment the labeled phosphate donor is $[^{32}P]$-ATP labeled in the gamma position.

In addition, compositions useful in specific detection and quantitation of DNA-

PK which comprise synthetic peptide substrates for use in the present method and kit

are provided. The synthetic peptide substrates are defined by the following features to

provide specific recognition and phosphorylation by DNA-PK: (1) a phosphate-

accepting amino acid pair which may include serine-glutamine (Ser-Gln) (SQ),

threonine-glutamine (Thr-Gln) (TQ), glutamine-serine (Gln-Ser) (QS), or glutamine-

threonine (Gln-Thr) (QT); (2) enhancer amino acids which may include glutamic acid

or glutamine immediately adjacent at the amino- or carboxyl- side of the amino acid

pair and forming an amino acid pair-enhancer unit; (3) a first spacer sequence at the

amino terminus of the amino acid pair-enhancer unit; (4) a second spacer sequence at

the carboxyl terminus of the amino acid pair-enhancer unit, which spacer sequences

may include any combination of amino acids that does not provide a phosphorylation

site consensus sequence motif; and (5) a tag moiety, which may be an amino acid

sequence or another chemical entity that permits separating the synthetic peptide from

the phosphate donor. Synthetic peptide substrates providing some or all of these features

include: Met Glu Glu Pro Gln Ser Asp Pro Ser Val Glu Pro Pro Leu Ser Gln Glu Thr Phe

Ser Asp Leu Trp Lys Leu Leu Pro Glu (SEQ ID NO: 1), Met Glu Glu Ser Gln Ser Asp Ile

Ser Leu Glu Leu Pro Leu Ser Gln Glu Thr Phe Ser Gly Leu Trp Lys Leu Leu Pro Pro

(SEQ ID NO: 2), Met Glu Glu Ser Gln Ser Asp Ile Ser Leu Glu Leu Pro Tyr Lys Lys

(SEQ ID NO: 3), Met Glu Glu Pro Gln Ser Asp Pro Ser Val Glu Pro Pro Leu Ser Gln Glu

Thr Phe Ser Asp Leu Trp Lys Lys (SEQ ID NO: 4), Asn Asn Val Leu Ser Pro Leu Pro

Ser Gln Ala Met Asp Asp Leu Met Lys Lys (SEQ ID NO: 6), Met Ala Ile Tyr Lys Gln

Ser Gln His Met Thr Glu Val Val Arg Arg (SEQ ID NO: 7), Glu Pro Pro Leu Ser Gln

Glu Thr Phe Ser Asp Leu Trp Lys Lys (SEQ ID NO: 8), Glu Pro Pro Leu Ser Gln Glu

Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 11), Glu Pro Pro Leu Ser Gln Glu Ala

Phe Ala Asp Leu Leu Lys Lys (SEQ ID NO: 12), Glu Pro Pro Gln Ser Leu Glu Ala Phe

Ala Asp Leu Trp Lys Lys (SEQ ID NO: 14), Glu Pro Pro Gln Ser Gln Glu Ala Phe Ala

Asp Leu Trp Lys Lys (SEQ ID NO: 15), Glu Pro Pro Leu Thr Gln Glu Ala Phe Ala Asp

Leu Trp Lys Lys (SEQ ID NO: 16), Glu Pro Pro Asp Ser Gln Glu Ala Phe Ala Asp Leu

Trp Lys Lys (SEQ ID NO: 17), Pro Glu Glu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys

Lys (SEQ ID NO: 18) and Pro Glu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ

ID NO: 19).

In a preferred embodiment the synthetic peptide substrate is selected from the

following synthetic peptides: Met Glu Glu Pro Gln Ser Asp Pro Ser Val Glu Pro Pro

Leu Ser Gln Glu Thr Phe Ser Asp Leu Trp Lys Leu Leu Pro Glu (SEQ ID NO: 1), Met

Glu Glu Ser Gln Ser Asp Ile Ser Leu Glu Leu Pro Leu Ser Gln Glu Thr Phe Ser Gly

Leu Trp Lys Leu Leu Pro Pro (SEQ ID NO: 2), Met Glu Glu Ser Gln Ser Asp Ile Ser

Leu Glu Leu Pro Tyr Lys Lys (SEQ ID NO: 3), Met Glu Glu Pro Gln Ser Asp Pro Ser

Val Glu Pro Pro Leu Ser Gln Glu Thr Phe Ser Asp Leu Trp Lys Lys (SEQ ID NO: 4),

Glu Pro Pro Leu Ser Gln Glu Thr Phe Ser Asp Leu Trp Lys Lys (SEQ ID NO: 8), Glu

Pro Pro Leu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 11), Glu

Pro Pro Leu Ser Gln Glu Ala Phe Ala Asp Leu Leu Lys Lys (SEQ ID NO: 12), Glu

Pro Pro Gln Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 15), Glu

Pro Pro Asp Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 17), Pro

Glu Glu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 18) , Pro Glu

Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 19), Pro Leu Ser Gln

Glu Thr Phe Ser Gly Leu Trp Lys Leu Leu Pro Pro-Lys Lys (SEQ ID NO: 63), and

Pro Leu Ser Gln Glu Ala Phe Ala Gly Leu Trp Lys Leu Leu Pro Pro-Lys Lys (SEQ ID

NO: 64).

In a most preferred embodiment the synthetic peptide substrate is selected from

the following peptides: Glu Pro Pro Leu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys

Lys (SEQ ID NO: 11), Glu Pro Pro Gln Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys

Lys (SEQ ID NO: 15), Pro Glu Glu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys

(SEQ ID NO: 18) and Pro Glu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ

ID NO: 19).

The method of the present invention can be used to detect the presence of DNA-

PK activity in a biological sample. The biological sample may be any eukaryotic cell

extract. For example, the cell extract may be prepared from human tissue culture cell

lines such as HeLa cells or 293 cells. In addition, rabbit reticulocytes, Sf9 cells, and

the eggs and oocytes of frogs (*Xenopus*), sea urchins (*Arbacia*), and clams (*Spisula*),

may also be used. Other biological samples may include extracts of primary tumors,

red blood cells, any other eukaryotic cell type and eukaryotic and prokaryotic cells

which have been engineered to express DNA-PK.

The DNA-PK may be secreted from the prokaryotic cell or may be found

internally in vesicles within the bacterial cell. Similarly, the eukaryotic cells may also

secrete DNA-PK or it may be found internally inside the cell. In each of these cases

either a cell-free supernatant or cell lysate may be used to assay for DNA-PK activity.

The synthetic substrate peptides of the present invention may also be used to detect

DNA-PK activity *in vivo* if the peptide is delivered to the interior of the cell by

microinjection or by similar means, or in cells permeabilized to permit entry of the

peptide into the cell.

In another embodiment of the present invention, the activity of a protein kinase

such as DNA-PK is monitored in living cells. The monitoring of protein kinase

activity, such as DNA-PK activity is partially accomplished by introducing to the cells

expression vectors containing genes encoding recombinant (artificial) protein substrates.

The protein substrates are expressed at moderate levels and are efficiently and specifically phosphorylated when a protein kinase such as DNA-PK is activated.

The typical artificial protein substrate, encoded by a recombinant gene of the present invention, consists of four functional elements, and two additional elements to facilitate analysis of the substrate *in vivo* and *in vitro*. The four functional components of these protein substrates are: (1) a protein segment containing a phosphorylation site consensus sequence motif for phosphorylation by a protein kinase or DNA-PK: (2) an optional nuclear localization signal; (3) an optional DNA-binding domain; and (4) an epitope that can be detected with high sensitivity after fractionation of cell extracts by polyacrylamide gel electrophoresis. The nuclear localization signal is believed to be required when the activity of nuclear kinases, such as DNA-PK, is being monitored. The DNA-binding domain is also believed to be required when the activity of DNA-PK is being monitored. The nuclear localization signal and DNA-binding domain may be omitted when monitoring protein kinases other than DNA-PK.

As previously described, the protein substrates encoded by the recombinant genes may also include two additional facilitory elements. These additional facilitory elements of the protein substrates are: (1) an epitope for affinity purification of the substrate; and (2) a site that permits cleavage (excision) of the segment of the protein substrate containing the phosphorylation site.

The expression vectors of the present invention are originally assembled in a host cell which does allow expression of the protein substrate. This is accomplished utilizing standard techniques known in the art. A suitable host for assembly of the expression vectors include *E. coli* strain BL21(DE3) (Novagen, 597 Science Drive,

Madison, WI 53711) and equivalents. Once assembled, the expression vectors are isolated from the host and can be introduced into other living cells. Once the expression vector is transferred, the modified cells can then be tested for expression of DNA-PK or other protein kinase activity.

After expression of the protein substrate, the amount of phosphorylated protein substrate is determined. The phosphorylated and non-phosphorylated protein substrates can be distinguished utilizing the techniques described previously for detecting the transfer of non-radioactive phosphate to a peptide substrate.

One preferred technique, which does not require radiolabeling of the phosphate or phosphoprotein, is to use isoelectrofocusing (IEF) polyacrylamide gel electrophoresis to separate the protein components of cell extracts which have been prepared in the presence of phosphatase and kinase inhibitors. Isoelectrofocusing separates proteins of different net charges. Accordingly, a phosphorylated protein which has a greater negative charge due to the added phosphate groups can be easily separated from its unphosphorylated protein counterpart. The proteins in the gel are then transferred from the gel to a membrane (e.g. nitrocellulose or polyvinylidene difluoride, PVDF) where the position(s) of the protein of interest is detected using an antibody that reacts with it.

If desired the position of the phosphorylated and non-phosphorylated substrates can be confirmed with radioactive precursors such as $^{32}PO_4$. This is accomplished by labeling the living cells with $^{32}PO_4$ prior to fractionation. The protein substrate can also be phosphorylated *in vitro* with $^{32}PO_4$. In either situation, after separation of the protein substrate and transfer to a membrane, the membrane is developed using autoradiographic procedures.

The protein substrate can also be partially purified utilizing the epitope tags for affinity purification if necessary. This is done if an insufficient amount of protein substrate required for easy detection is not expressed in the cells, or if removal of proteins other than the protein substrate is desired. In a preferred embodiment, the epitope is a sequence of amino acids which allow immobilized metal affinity purification. In a most preferred embodiment, six consecutive histidine (His) residues are inserted after the N-terminal phosphorylation site segment to provide the epitope. This allows purification with immobilized metals such as Nickel (Ni) or Zinc (Zn).

The determination of the amount of phosphorylated protein substrate is correlated to the concentration of either protein kinase or DNA-PK activity; any of the methods described previously for determining activity are applicable.

In a preferred embodiment of the method of monitoring DNA-PK activity in a cell, recombinant DNA encoding the protein substrates 1 and 4 shown in Figure 4, is utilized. The recombinant gene encoding protein substrate 1 is most preferred. The insertion of one of these prototype recombinant genes in an appropriate expression vector system, e.g. the T7-derived plasmid expression vector, pET28 enables the encoded proteins to be efficiently produced in *Escherichia coli*.

All six protein substrates shown in Figure 4 contain the following elements: (1) a phosphorylation site concensus sequence motif for DNA-PK; (2) a DNA-binding domain; (3) a detection epitope tag; (4) an epitope of six consecutive histidine residues for immobilized metal-affinity purification; and (5) a consensus cleavage site located after the histidine tag.

The phosphorylation site for DNA-PK is contained within the sequence Met Pro Glu Glu Ser Gln Glu Thr Phe Glu Asp Leu Trp Lys Leu Leu Pro (SEQ ID NO: 40) in which serine residue 5 is phosphorylated by DNA-PK *in vitro*. This sequence is comprised of the first four residues of human hsp90, Met Pro Glu Glu (SEQ ID NO: 41), and a variant of residues 15 to 27 of human p53, Ser Gln Glu Thr Phe Ser Asp Leu Trp Lys Leu Leu Pro (SEQ ID NO: 42). The substitution of glutamic acid (Glu) for the second serine (Ser) at the position of serine 20 in human p53 allowed the introduction of a *Bgl* II restriction endonuclease cloning site in the substrate gene which facilitates the construction of genes with different phosphorylation sites. With a few exceptions, (SEQ ID NO: 40) is also identical to the synthetic peptide substrate Pro Glu Glu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 18). First, the synthetic peptide substrate lacks the amino-terminal methionine (Met) which is expected to be removed *in vivo* (Lees-Miller, et al., 1989, *J. Biol. Chem.*, 264:17275-17280). Second, threonine (Thr) at the position of threonine 18 of human p53 protein and serine (Ser) at the position of serine 20 of human p53 protein are replaced by alanine in the synthetic peptide substrate (SEQ ID NO: 18).

The DNA-binding domain is the wild-type human POU domain (residues Glu[280]-Pro[439]) of the human Oct-1 transcription factor (Genbank accession no. X13403 (HSOCT1), human mRNA for octamer-binding protein Oct-1, 2584 bp). Sequences within this domain also act as nuclear localization signals. A mutant POU domain which lacks a natural protein kinase A (PKA) site at Ser[285] in the human Oct-1 protein also may be used.

The detection epitope tag is the sequence Glu Pro Glu Leu Ala Pro Glu Asp Pro

Glu Asp (SEQ ID NO: 43), which corresponds to a segment (residues 289-299) from

the Herpes simplex virus 1 glycoprotein D precursor, and is derived from the Novagen

pET-25b vector.

A series of six consecutive histidines are inserted after the N-terminal

phosphorylation site segment to provide an epitope for immobilized metal-affinity

purification. Thus, partial purification of the expressed protein substrate can be

accomplished through the utilization of this tag.

Finally, a consensus cleavage site Met Ser Gly Gly (SEQ ID NO: 44) for the

human adenovirus endoproteinase (C.W. Anderson, 1993, *Protein Express. Purif.*: 4:8-

15) is located after the histidine tag.

The predicted primary sequence of the most preferred protein substrate, the

wild-type artificial substrate (substrate 1 in Figure 4) is shown in Figure 8B.

The kit to monitor protein kinase or DNA-PK activity within a cell includes the

expression vector described in the method of the invention. Accordingly, the

appropriate protein substrate encoding gene is incorporated into the expression vector to

provide the desired phosphorylation site consensus sequence motif for either the protein

kinase or DNA-PK. With the kit for monitoring DNA-PK activity, an agent that nicks

or otherwise damages DNA may also be included to provide short DNA segments to

activate DNA-PK within the cell. Purified samples of the protein substrates, and their

phosphorylated counterparts, may also be included as controls. An appropriate reagent

to determine the amount of phosphorylated peptide substrate following expression in the

cell may also be included in the kit. Such reagents may include: (1) monoclonal

antibodies to detect the substrate tag; (2) sources or preparations of DNA-PK and

DNA; (3) materials for polyacrylamide gel electrophoresis and/or IEF gel

electrophoresis; and (4) materials for blotting the protein substrate on nitrocellulose and

PVDF membranes.

The method for identifying agents that alter either intracellular protein kinase or

intracellular DNA-PK activity is identical to the method of monitoring activity except

for an additional step. The cells are contacted with agents that may alter protein kinase

or DNA-PK activity. This procedure is basically the same as in the method for

detecting substances that alter the activity DNA-PK *in vitro*. In a second embodiment

of the method, cells may be treated with an agent that activates the protein kinase or

DNA-PK, then the cells are contacted, either before or after, with an agent that may

inhibit activation of the protein kinase or DNA-PK. The amount of phosphorylated

substrate in the treated cells is then compared to the amount of phosphorylated substrate

in control cells, i.e., non-contacted cells containing the protein substrates.

The monitoring of intracellular protein kinase activity and DNA-PK activity is

also accomplished through the expression of a reporter gene product in a transfected or

transformed cell. This is partially accomplished by introducing into a living cell an

expression vector, such as those previously described, with one modification. The

expression vector will contain a gene coding for a phosphorylatable protein substrate

capable of activating the transcription of a reporter gene. By determining the amount

of reporter gene product expressed, a correlation to the concentration of protein kinase

or DNA-PK activity can be accomplished. Alternatively, the expression of the reporter

gene product can be used in a qualitative manner to determine if protein kinase or

DNA-PK activity exists.

As previously described, the phosphorylation of the protein substrate activates

the expression of the reporter gene product whose synthesis and activity can be

determined or for which a qualitative selection mechanism is available (e.g. confers

antibiotic resistance). Common reporter genes are β-galactosidase, luciferase, the gene

for neomycin resistance, or the DHFR gene for MTX resistance.

The protein substrate for this embodiment of the present invention has the

following elements to facilitate transcription of the reporter gene: (1) a transactivation

domain which contains a phosphorylation site consensus sequence motif specific for

either a protein kinase or DNA-PK; and (2) a DNA binding domain. The activation of

the transactivation domain by phosphorylation thereby activates the transcription of the

reporter gene.

As known in the art, the activation of transactivation domain can be

accomplished directly or indirectly via phosphorylation. In the preferred embodiment

of the present invention, activation of the transactivation domain is achieved indirectly

via phosphorylation. In the most preferred embodiment of the present invention,

indirect activation of the transactivation domain is achieved via phosphorylation by

DNA-PK.

The most preferred protein substrate for DNA-PK phosphorylation includes as

the transactivation domain residues 1-73 of p53 and as the DNA binding domain

residues 1-147 of gal4. This embodiment of the invention is based on the assumption

that phosphorylation of the DNA-PK site in the transactivation domain of the human

p53 protein regulates its interaction with the Hmd2 protein. Hmd2 is the product of a

cellular proto-oncogene that has been shown to regulate (i.e., inactivate) the

transcriptional activity of p53 by binding to a site near the amino terminus of p53. The

Hmd2 binding site is located adjacent to the serine 15 phosphorylation site for DNA-PK

(Chen et al., 1994, *Proc. Natl. Acad. Sci. USA*, 91;2684-2688; Haines et al., 1994,

Mol. Cell. Biol., 14:1171-1178; Lin et al., 1994, *Genes Dev.*, 8:1235-1246; Momand

et al., 1992, *Cell*, 69:1237-1245; Oliner et al., 1993, *Nature*, 362:857-860; Wu et al.,

1992, *Genes Dev.*, 7:1126-1132; D. Lane, personal communication).

As known in the art, the phosphorylation of human p53 at amino terminal

serines including serine 15 by DNA-PK is believed to inhibit Hmd2 binding to p53,

thereby activating p53-mediated transcription. Similarly, it is believed that the

activation of transcription of a reporter gene can be achieved with the artificial

recombinant transcription factor of the present invention, which includes the

transactivation region of p53, residues 1-73 of human p53 (Unger et al., 1992, *EMBO

J.*, 11:1383-1390; A.J. Levine, 1993, *Ann. Rev. Biochem.*, 62:623-651), attached to an

appropriate DNA-binding domain, e.g. residues 1-147 of the yeast gal4 protein.

In cells that overexpress Hmd2, whether naturally or through genetic

engineering, the artificial recombinant transcription factor will remain inactive because

of its complex formation with Hmd2. Upon activation of DNA-PK and other protein

kinases, the recombinant transcription factor will be phosphorylated and activated,

which, in turn, will activate transcription of the reporter gene.

As will be apparent to one skilled in the art, variations of the most preferred

embodiment are possible. However, the protein substrate must efficiently bind Hmd2

(or an equivalent factor) and phosphorylation of the protein substrate must inhibit this interaction.

In a second embodiment of this method, the activation of the transactivation domain is achieved directly via phosphorylation of the protein substrate. The direct activation of the protein substrate provides a simpler mechanism for transcription of the reporter gene since an inhibiting protein, such as Hmd2, is not present.

The preferred protein substrate for this embodiment contains the residues of the protein c-Jun, which is found in eukaryotic cells. C-Jun is composed of two functional domains, a DNA-binding domain located near its carboxyl-terminus and a transactivation domain near its amino terminus (Angel, et al., 1989, *New Biol.*, 1:35-43; Bohmann, et al., 1989, *Cell*, 59:709-711; Smeal, et al., 1989, *Genes & Dev.*, 3:2091-2100). It has been found that the phosphorylation of serine 63 and serine 73 in the amino-terminal activation domain of the protein c-Jun potentiates its transactivation function (Hibi, et al., 1993, *Genes & Dev.*, 7:2135-2148). Accordingly, expression vectors containing the protooncogene c-Jun, or a modified version thereof, can be constructed and utilized in accordance with the present invention. For example, the sequence of the c-Jun transactivation region would be modified around the serine 63 and 73 sites to make these sites specific for DNA-PK.

EXAMPLES

The following examples are provided to further illustrate the present invention. Examples 1-8, are provided to illustrate protocols in preparing the materials needed for the present invention. Examples 9-14 specifically illustrate preferred embodiments and methods of using the present invention. Examples 15-17 illustrate the preferred

additional embodiments of the present invention utilizing expression vectors which encode the recombinant protein substrates for DNA-PK.

Example 1

Preparation of Cell Extracts

Those skilled in the art recognize that many methods exist to prepare cell extracts and cell free supernatants. The method used will depend upon the source of the starting material. For example, if eukaryotic tissue culture cells are used they are grown in an appropriate nutrient medium. Cells are usually grown to a concentration of approximately 0.7 to 1 X 10^6 cells per milliliter, however, optimal concentrations will vary according to the cell line employed as well as other parameters known in the art. Furthermore, the health and viability of the cells is more important than cell density.

Once the cell cultures are ready to be harvested, cell extracts can be prepared by any of several methods known to those skilled in the art in order to provide a solution containing DNA-PK. In particular, cytoplasmic extracts of HeLa and mouse cells can be prepared from cells grown in suspension culture to approximately 5 x 10^5 cells/ml. Cells are harvested by centrifugation at 4°C. After three washes in ice-cold phosphate buffered saline (PBS), the cell pellet is resuspended in one packed cell volume of ice-cold low salt buffer (LSB: 25 mM KCl, 10 mM NaCl, 1.1 mM $MgCl_2$, 0.1 mM EDTA, 10 mM HEPES, pH 7.2 with KOH at 20°C), and allowed to swell for 10 min. Cells are broken by approximately 20 strokes in a tight fitting stainless steel homogenizer (Wheaton Instruments, Millville, NJ).

An S10 supernatant was prepared from the homogenate by centrifugation at

10,000 g for 30 min at 4°C. Supernatants were stored frozen in liquid nitrogen in

small aliquots. Typrical extracts may have an absorbance at 260 nm of between 50 and

100. To prepare extracts from small numbers of cells (e.g., approximately 5×10^6 - 5

x 10^7) the homogenization step was replaced by a single freeze-thaw cycle. Other cell

types were also prepared in a similar fashion. For example, rabbit reticulocyte lysates

can be prepared as described by Jackson, et al., in *Methods Enzymol.*, 96:50-74 (1983).

Extracts of *Arbacia* eggs can be prepared as described by Ballinger, et al., in *Dev.*

Biol., 101:192-200 (1984).

Example 2

Preparation of DNA-PK, mouse p53, hsp 90 and oligonucleotides

DNA-PK was purified from HeLa cells as described previously by Lees-Miller,

et al., *Mol. Cell. Biol.*, 10:6472-6481 (1990) and as modified in Lees-Miller, et al.,

Mol. Cell. Biol., 12:5041-5049 (1992). In particular, extracts of HeLa S3 cells were

prepared by a single freeze-thaw cycle of a cell suspension as described in Example 1

and reported by Lees-Miller, et al., *J. Biol. Chem.*, 264:2431-2437 (1989).

Phenylmethylsulfonyl fluoride was added to the S10 (10,000 x g) supernatant as a

powder to 0.5 mM. DNA-PK activity was found in the 100,000 x g (ribosomal) pellet.

DNA-PK preparations typically were made from 200 ml of S10 supernatant that had

been derived from approximately 10^{10} cells. All steps were performed at 4°C unless

indicated otherwise.

The S100 ribosomal pellet containing DNA-PK activity was solubilized by

sonicating twice for 5 seconds each time in the water-cooled horn of a Branson Sonifier

(Branson Sonic Power Co., Danbury, CT) in 40 ml of 25 mM N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (HEPES)-0.2 mM EDTA-0.5 mM dithiothreitol (pH 7.5) (HEPES buffer) containing 0.5 M KCl and 10 mM $MgCl_2$. After recentrifugation at 100,000 x g for 60 min, the ribosomal salt wash can be stored frozen at -70°C. The salt wash was diluted with HEPES buffer to give a KCl concentration of 0.1 M, centrifuged for 15 min at 10,000 x g to remove precipitates, and applied to a column (2.5 by 5 cm) of DEAE-Sepharose CL-6B (Sigma Chemical Co., St. Louis, MO) equilibrated in HEPES buffer containing 0.1 M KCl. After being washed, the column was eluted with HEPES buffer containing 0.5 M KCl. This eluate was dialyzed for 1 hour against 10 volumes of HEPES buffer containing 50 mM KCl and adjusted to 0.1 M KCl before application to a column (1.5 by 4 cm) of dsDNA-cellulose (Sigma Chemical Co., St. Louis, MO) equilibrated in HEPES buffer with 0.1 M KCl. The fraction containing DNA-PK activity was eluted from dsDNA-cellulose with either a step of 0.5 M KCl or a 30-ml linear gradient from 0.1 to 0.5 M KCl in HEPES buffer. The fraction containing DNA-PK activity eluted from dsDNA-cellulose at a KCl concentration of between 0.2 and 0.4 M KCl. Active fractions containing DNA-PK were pooled, aliquoted, and stored at -70°C. At this step, the fraction containing DNA-PK activity was stable for several months, and aliquots could be frozen and thawed numerous times.

Active fractions containing DNA-PK were further purified by chromatography on DEAE-Sepharose in the presence of 25 mM $MgCl_2$, on single-stranded DNA (ssDNA)-cellulose, on Mono S Fast Flow (Pharmacia-LKB Biotechnology, Piscataway, NJ), on phosphocellulose (Sigma Chemical Co., St. Louis, MO), on heparin-Sepharose

(Sigma Chemical Co., St. Louis, MO) or on ATP-agarose type 4 (Pharmacia-LKB

Biotechnology, Piscataway, NJ). The first three procedures were used to show that

hsp90, SV40 TAg, and p53 were phosphorylated by the same activity. The dsDNA-

cellulose eluate was adjusted to 0.1 M KCl by a brief dialysis as described above,

$MgCl_2$ was added to 25 mM, and the extract was applied to a column (2.5 by 4 cm) of

DEAE-Sepharose CL-6B (Sigma Chemical Co., St. Louis, MO) equilibrated in 0.1 M

KCl-HEPES buffer containing 25 mM $MgCl_2$. Under these conditions, DNA-PK

activity did not bind to the column, and the flow through containing DNA-PK was

applied directly to a column (1.5 by 4 cm) of ssDNA-cellulose equilibrated in HEPES

buffer containing 0.1 M KCl. DNA-PK activity was eluted with a 16-ml linear gradient

from 0.1 to 0.5 M in KCl at a flow rate of 0.5 ml/min. Active fractions containing

DNA-PK were pooled, dialyzed for 1 h, and applied to a column (1 by 1 cm) of Mono

S Fast Flow. This column was eluted with a 10-ml linear gradient of 0.1 to 0.3 M KCl

in HEPES buffer. Active fractions containing DNA-PK were again pooled and then

concentrated by using a Centricon 30 unit (Amicon Corporation, Lexington, MA).

DNA-PK preparations purified from human tissue culture cells through dsDNA-

cellulose have a specific activity of approximately 3 to 6 U/mg of protein;

approximately 6 mg of protein (Bio-Rad assay) is obtained from 10^{10} cells. DNA-PK

purified through phosphocellulose or Mono S Fast Flow has a specific activity of up to

200 U/mg, and protein yield was about 5 μg per 10^{10} cells. A unit of activity is defined

as 1 nmol of phosphate transferred to hsp90 per min at 30°C under the assay conditions

described in Example 4. Highly purified DNA-PK loses activity when stored on ice or

frozen at -70°C.

In current procedures the gradient elution from Q-Sepharose Fast Flow

described above was substituted for DEAE-Sepharose chromatography. In addition, the

gradient elution from S-Sepharose Fast Flow (Pharmacia-LKB Biotechnology,

Piscataway, NJ) described above was substituted for double-stranded DNA-cellulose

chromatography. The present procedure achieved a similar DNA-PK specific activity

as described above but yielded more activity per cell.

Wild-type mouse p53 was purified by immunoaffinity chromatography from Sf9

insect cells infected with the recombinant virus NPVp53 (Stenger, et al. 1992, *Mol.*

Carcinog., 5:102-106). Human hsp90 was purified, as reported by Lees-Miller, et al.,

J. Biol. Chem., 264:2431-2437 (1989). Alternating $(dG-dC)_n$ oligonucleotides of

defined length were synthesized on a Milligen/Biosearch 8750 oligonucleotide

synthesizer and purified by using an RP-1 cartridge (Glen Research, Co., Sterling,

VA); before use, the oligonucleotides were dissolved at 1 mg/ml in 10 mM Tris-HCl

(pH-8)-1 mM EDTA-50 mM NaCl, heated briefly at 100°C, and allowed to cool

slowly overnight (Lees-Miller, et al., 1992, *Mol. Cell. Biol.*, 12:5041-5049).

Example 3

Preparation of synthetic peptides

It was previously demonstrated that two sites of the heat shock protein hsp90

and four sites of the SV40 TAg protein could be phosphorylated *in vitro* by DNA-PK

(Lees-Miller, et al., 1989, *J. Biol. Chem.*, 264:17275-17280, and Chen, et al., 1991,

J. Virol. 10:5131-5140). Each phosphorylation site was a threonine or serine residue

that was immediately followed by a glutamine residue, suggesting Ser-Glu (SQ) or Thr-

Glu (TQ) was required for *in vitro* phosphorylation by DNA-PK. It was suggested that

SQ and TQ might represent the phosphorylation site consensus sequence motif for

DNA-PK. The following experimental work was undertaken to determine the accuracy

of that suggestion and to determine the other structural requirements for synthetic

peptides that were substrates for DNA-PK that provided high specificity and excellent

kinetic properties.

Wild-type human p53 has six serines (but no threonines) that are immediately

preceded or followed by glutamine: serines 6 (Gln-Ser-Asp), 15 (Leu-Ser-Gln), 37

(Pro-Ser-Gln), 99 (Pro-Ser-Gln), 166 (Gln-Ser-Gln), and 376 (Gln-Ser-Thr) (Lees-

Miller, et al., 1992, *Mol. Cell. Biol.*, 12:5041-5049). To determine whether these sites

could be phosphorylated by DNA-PK, peptides were synthesized corresponding to

sequences surrounding these serines, (SEQ ID NO: 4), (SEQ ID NO: 6), (SEQ ID NO:

28), (SEQ ID NO: 7), and (SEQ ID NO: 30) (see Table 1). In addition, peptides were

synthesized that contained the p34[cdc2] kinase site at serine 312 (SEQ ID NO: 29) and

that contained the casein kinase II site, at serine 392 (SEQ ID NO: 31). Additional

synthetic peptides which were tested for use as DNA-PK-specific substrates are listed in

Table 1. For most peptides that had fewer than two arginine or lysine residues, one or

two lysines were added at the carboxy terminus to facilitate peptide binding to

phosphocellulose paper (Glass, et al., 1978, *Anal. Biochem.*, 87:566-575).

Protected peptide chains were assembled by the stepwise solid-phase method,

using a 430A Automated Peptide Synthesizer (Applied Biosystems Inc., Foster City,

CA). After removal of the N-tert-butoxycarbonyl (tBoc) group with trifluoroacetic

acid, cleavage of the peptide from the resin and removal of side chain protecting groups

were accomplished with HF in the presence of p-cresol. The crude peptide was

extracted with 5% acetic acid and chromatographed on Sephadex G-25 (Sigma

Chemical Co., St. Louis, MO). Further purification was by reverse-phase high-

pressure liquid chromatography (HPLC) on a Vydac C_4 column (The Nest Group,

Southboro, MA) in 0.05% trifluoroacetic acid-water-acetonitrile.

(SEQ ID NO: 12) (Table 1) and the synthetic casein kinase I substrate Asp Asp

Asp Glu Glu Ser Ile Thr Arg Arg (SEQ ID NO: 35), described by Agostinis, et al.,

FEBS Lett., 259:75-78 (1989) were obtained from (Multiple Peptide Systems, Inc, San

Diego, CA). The S6 kinase substrate Arg Arg Leu Ser Ser Leu Arg Ala (SEQ ID NO:

36) was purchased from (Bachem California, Inc., Torrance, CA); the casein kinase II

substrate Arg Arg Arg Glu Glu Glu Thr Glu Glu Glu (SEQ ID NO: 37) was provided

by E. Krebs, University of Washington, Seattle. The purity of each peptide, as judged

from its reverse-phase HPLC profile, was greater than 95%. Purified peptides were

dissolved in water and stored at -20°C. Peptide concentrations were determined by

quantitative amino acid analysis after hydrolysis for 24 h at 105°C in 6 N HCl. Each

peptide was tested as a potential DNA-PK substrate.

Table 1

Synthetic p53 Peptide Substrates

SEQ NO NO:	p53 residues[a]	Peptide Sequence[b]	K_m (µM)	V_{max}(pmol/ min/µg of DNA-PK	PO₄ incorp. (pmol/min/µg of DNA-PK)[c]
1	1-28	Met Glu Glu Pro Gln Ser Asp Pro Ser Val Glu Pro Pro Leu **Ser Gln** Glu Thr Phe Ser Asp Leu Trp Lys Leu Leu Pro Glu	ND[d]	ND	ND
2	4-31	Met Glu Glu **Ser Gln** Ser Asp Ile Ser Leu Glu Leu Pro Leu **Ser Gln** Glu Thr Phe Ser Gly Leu Trp Lys Leu Leu Pro Pro	ND*	ND*	ND*
3	4-13	Met Glu Glu **Ser Gln** Ser Asp Ile Ser Leu Glu Leu Pro-Tyr Lys Lys	650	390	94
4	1-24	Met Glu Glu Pro Gln Ser Asp Pro Ser Val Glu Pro Pro Leu **Ser Gln** Glu Thr Phe Ser Asp Leu Trp Lys-Lys	210	90	44
5	1-24	Met Glu Glu Pro Gln Ser Asp Pro Ser Val Glu Pro Pro Leu Ala Gln Glu Thr Phe Ser Asp Leu Trp Lys-Lys	--[f]	--	5.3
6	29-44	Asn Asn Val Leu Ser Pro Leu Pro **Ser Gln** Ala Met Asp Asp Leu Met-Lys Lys	470	470	102
28	92-108	Pro Leu Ser Ser Ser Val Pro **Ser Gln** Lys Thr Tyr Gln Gly Ser Tyr Gly Lys Lys	--	--	3
7	160-175	Met Ala Ile Tyr Lys Gln **Ser Gln** His Met Thr Glu Val Val Arg Arg	740	200	44
29	306-327	Ala Leu Pro Asn Asn Thr Ser Ser Ser Pro Gln Pro Lys Lys Lys Pro Leu Asp Gly Glu Tyr	--	--	0.6
30	371-385	Ser Lys Lys Gly Gln Ser Thr Ser Arg His Lys Lys Leu Met Phe	--	--	0
31	380-393	His Lys Lys Leu Met Phe Lys Thr Glu Gly Pro Asp Ser Asp	--	--	0
8	11-24	Glu Pro Pro Leu **Ser Gln** Glu Thr Phe Ser Asp Leu Trp Lys-Lys	350	360	130
9	11-19	Glu Pro Pro Leu **Ser Gln** Glu Thr Phe-Lys Lys	ND	ND	15
10	11-20	Glu Pro Pro Leu **Ser Gln** Glu Thr Phe Ser Asp-Lys Lys	ND	ND	12

Table 1 (continued)

Synthetic p53 Peptide Substrates

SEQ NO NO:	p53 residues[a]	Peptide Sequence[b]		K_m (µM)	V_{max}(pmol/ min/µg of DNA-PK	PO4 incorp. (pmol/min/µg of DNA-PK)[c]
11	11-24[e]	Glu Pro Pro Leu **Ser Gln** Glu _Ala_ Phe	_Ala_ Asp Leu Trp Lys-Lys	760	380	83
12	11-24[e]	Glu Pro Pro Leu **Ser Gln** Glu _Ala_ Phe	_Ala_ Asp Leu Leu Lys-Lys	560	160	36
32	11-24[e]	Glu Pro Pro Leu Ser _Glu_ Glu _Ala_ Phe	_Ala_ Asp Leu Trp Lys-Lys	ND	ND	0.4
33	11-24[e]	Glu Pro Pro Leu Ser _Asn_ Glu _Ala_ Phe	_Ala_ Asp Leu Trp Lys-Lys	ND	ND	0.1
20	11-24[e]	Glu Pro Pro Leu Ser _Glu_ _Gln_ _Ala_ Phe	_Ala_ Asp Leu Trp Lys-Lys	ND	ND	0
13	11-24[e]	Glu Pro Pro Leu **Ser Gln** _Lys_ _Ala_ Phe	_Ala_ Asp Leu Trp Lys-Lys	1,000	70	14[g]
14	11-24[e]	Glu Pro Pro _Gln_ **Ser** _Leu_ Glu _Ala_ Phe	_Ala_ Asp Leu Trp Lys-Lys	420	310	99
15	11-24[e]	Glu Pro Pro _Gln_ **Ser Gln** Glu _Ala_ Phe	_Ala_ Asp Leu Trp Lys-Lys	290	390	161
16	11-24[e]	Glu Pro Pro Leu **Thr Gln** Glu _Ala_ Phe	_Ala_ Asp Leu Trp Lys-Lys	670	460	116
17	11-24[e]	Glu Pro Pro _Asp_ **Ser Gln** Glu _Ala_ Phe	_Ala_ Asp Leu Trp Lys-Lys	ND	ND	48
18	12-24[e]	_Pro_ _Glu_ _Glu_ **Ser Gln** Glu _Ala_ Phe _Ala_	Asp Leu Trp Lys-Lys	270	410	180
19	14-24[e]	_Pro_ _Glu_ **Ser Gln** Glu _Ala_ Phe _Ala_ Asp	Leu Trp Lys-Lys	200	470	220
34	11-24[e]	Glu Pro Pro Leu Ser _Tyr_ Glu _Ala_ Phe	Ala Asp Leu Leu Lys Lys	ND	ND	2.3

[a]The amino acid positions corresponding to the first and last p53 residues in each peptide are given; all sequences are identical to or variants of human p53 protein except (SEQ ID NO: 2) and (SEQ ID NO: 3), which correspond to mouse p53 protein.

[b]The sequence is given in the three-letter amino acid code; serines (Ser) followed by glutamine (Gln) are shown in bold type; changes from the wild-type sequence of p53 to produce the variant peptides are underlined; lysines (Lys) added at the carboxy terminus are separated by a dash.

[c]Phosphate incorporated for each peptide at 200 µM.

[d]ND, not determined

[e]Differs from the wild-type p53 sequence and represents a variant of human p53 N terminal sequences

[f]--, an appropriate concentration range for determining the indicated value was not achieved.

ᵍObtained at 300 µM peptide

*See below for analysis of phosphorylation reactions

Example 4

Phosphorylation reactions

Phosphorylation reactions were performed in a 20- or 40-µl final volume as

previously described by Chen, et al., *J. Virol.*, 10:5131-5140 (1991) and Lees-Miller,

et al., *Mol. Cell. Biol.*, 10:6472-6481 (1990). In particular, in addition to synthetic

peptide substrates or protein substrates, the assays contained 50 mM HEPES (pH 7.5),

100 mM KCl, 10mM $MgCl_2$, 2 mM EGTA, 0.1 mM EDTA and 0.125 mM ATP

containing [^{32}P]ATP at 0.2 mCi/ml. Sonicated calf thymus DNA was added to 10

µg/ml. Assays were started by adding 1 µl of kinase sample. Incubations were at

30°C for 7 min.

For determinations of kinetic constants, the ATP concentration was 0.5 mM.

When present, hsp90 was included at 0.5 mg/ml. Assay mixtures were preincubated at

30°C for 2 min before addition of 0.1 to 0.5 µg (protein) of the DNA-PK preparation.

Example 5

Assay of synthetic peptides

The transfer of [^{32}P]phosphate to synthetic peptides was quantitated by the

phosphocellulose paper binding method described by Casnellie, *Methods Enzymol.*,

200:115-120 (1991) and Glass, et al., *Anal. Biochem.*, 87:566-575 (1978). In

particular, kinase reactions were stopped by adding an equal volume of 30% acetic

acid, and portions of the acidified reaction were spotted on 2- by 2-cm squares of P-81

paper (Whatman, Inc., Clifton, NJ). Squares were washed four times for 5 min each in

15% acetic acid. The P-81 squares were then transferred to scintillation vials

containing 3.5 ml of water, and bound radioactivity was determined from the Cerenkov

radiation. Enzyme activity was calculated from triplicate determinations, using the

specific activity of the ATP and the protein concentration, which was determined by the

Bio-Rad dye-binding assay (Bio-Rad (Bradford) Protein Assay Kit 1, Cat. No. 500-001,

Bio-Rad Laboratories, Hercules, CA), using bovine serum albumin as the standard.

Blank values, obtained without added peptide, gave less than 0.5 pmol of bound

phosphate; these blanks were subtracted from assay values with peptides. Control

assays indicated that at least 80% of spotted phosphopeptides bound to P-81 squares and

that the fraction bound was linear over the concentration range of peptide used. The

kinetic constants, K_m and V_{max} for each peptide sequence were calculated from plots of

1/[S] versus 1/V, the values reported are averages of at least two independent

determinations. These results, including the quantitation of [^{32}P] phosphate transfer for

synthetic peptide substrates, are shown in Table 1.

Phosphorylation of peptides that lacked positively charged residues was

estimated from gel filtration profiles. Peptides were separated from [^{32}P]ATP by

passage through a column (1.5 by 12 cm) of Bio-Gel P4 (Bio-Rad Laboratories,

Hercules, CA) in 10% acetic acid.

Example 6

20   **Analysis of phosphorylation of synthetic peptides by DNA-PK**

As shown in Table 1, only the peptides derived from human p53 sequence

containing serines 6 and 15 (SEQ ID NO: 4), serine 15 (SEQ ID NO: 8), serine 37

(SEQ ID NO: 6), and serine 166 (SEQ ID NO: 7) were phosphorylated at a significant

rate at the concentrations studied. The K_m for (SEQ ID NO: 4), Met^1-Lys^{24}-Lys (the lysine added at the carboxy terminus is not part of the p53 sequence), containing both serines 6 and 15 was 210 μM, and V_{max} was 90 pmol/min/μg. The K_m for (SEQ ID NO: 8), Glu^{11}-Lys^{24}-Lys was approximately fifty percent higher (360 μM), but its V_{max} was four-fold greater (360 pmol/min/μg of DNA-PK). The K_m for (SEQ ID NO: 6), Asn^{29}-Met^{44}-Lys-Lys, was twice as high, but its V_{max} was five times greater. (SEQ ID NO: 7), Met^{160}-Arg^{175}, was phosphorylated at less than half of the rate of (SEQ ID NO: 6), when both were assayed at 200 μM, but it was active as a substrate for DNA-PK. At 200 μM, the rate of phosphorylation of (SEQ ID NO: 28), Pro^{92}-[Ser^{99}]-Gly^{108}-Lys-Lys, was less than 7% of the rate at which (SEQ ID NO: 4) was phosphorylated. Three other peptides (SEQ ID NOS: 29, 30 and 31) were also phosphorylated at a negligible rate by DNA-PK. In all cases in which phosphorylation of the peptide substrate was observed, phosphorylation was completely dependent on the presence of added dsDNA. These results clearly demonstrated that all Ser-Gln sequences are not phosphorylated by DNA-PK and that the consensus sequence motif for DNA-PK must include additional amino acid residues.

(SEQ ID NO: 7), Met^{160}-Arg^{175}, has only one serine; thus, the serine equivalent to p53 residue 166 must be phosphorylated. (SEQ ID NO: 6), Asn^{29}-Met^{44}-Lys-Lys, has two serines, equivalent to residues 33 and 37 of human p53. To determine which of these were phosphorylated, after incubation with DNA-PK, radiochemical sequence analysis was performed by using a Beckman 890M spinning-cup sequencer (Beckman Instruments, Palo Alto, CA) as described by Lees-Miller, et al., *J. Biol. Chem.*, 264:17275-17280 (1989). The peptide was isolated by reverse-phase HPLC,

peptidylphosphoserine was converted to S-ethylcysteine, and the position of

phenylthiohydantoin (PTH)-S-ethylcysteine was determined by chemical microsequence

analysis using an Automated Protein/Peptide sequencer (Applied Biosystems, Foster

City, CA) and a 120 PTH-Amino Acid Analyzer (Applied Biosystems, Foster City,

CA) as described by Lees-Miller, et al., *J. Biol. Chem.*, 264:17275-17280 (1989).

Only the ninth residue, i.e., equivalent to serine 37, had a significant amount of PTH-

S-ethylcysteine, indicating that serine 37 was phosphorylated by DNA-PK whereas the

equivalent to serine 33 was not phosphorylated. This result clearly demonstrates that

the phosphate-accepting serine must be immediately adjacent to a glutamine, *i.e.*, either

Ser-Gln or Gln-Ser. In addition, peptides (SEQ ID NOS: 29 and 31) which do not

contain a serine immediately adjacent to a glutamine are not substrates for DNA-PK.

The amino-terminal peptide of human p53 protein, Met^1-Lys^{24}-Lys, (SEQ ID

NO: 4), has four serine residues. Radiochemical sequence analysis showed that the

major site of phosphorylation was serine 15. To verify this result, in peptide (SEQ ID

NO: 5), Ser^{15} was replaced with alanine, an unphosphorylatable amino acid. This

replacement caused the peptide to become an ineffective substrate for DNA-PK. At

200 μM, (SEQ ID NO: 5), Met^1-$[Ala^{15}]$-Lys^{24}-Lys, was phosphorylated at about 10% of

the rate of the wild-type peptide sequence, confirming that serine 15 was the major site

of phosphorylation of (SEQ ID NO: 4) (Table 1). This conclusion was also confirmed

with (SEQ ID NO: 8), Glu^{11}-$[Ser^{15}]$-Lys^{24}-Lys (and other peptide variants of this amino

terminal peptide sequence), lacking serines 6 and 9. (SEQ ID NO: 8) was

phosphorylated at four times the rate of the (SEQ ID NO: 4), Met^1-Lys^{24}-Lys wild-type

peptide, but its K_m was 1.5 times higher (Table 1).

A peptide corresponding to mouse p53 residues Met^4-Pro^{31}, (SEQ ID NO: 2),

was also examined (See Table 1). Serine 7 of mouse p53 is at the position equivalent to

proline 4 of human p53, and the glutamine at position 8 of mouse p53 is conserved

among mammals as reported by Soussi, et al., *Oncogene*, 5:945-952 (1990). After

phosphorylation of (SEQ ID NO: 2) the peptide was digested with endoproteinase Glu-

C, and the resulting fragments were separated by reverse-phase HPLC. Two major

phosphopeptides were obtained, and sequence analyses indicated that one began at Met^1

and the other began at Leu^{15}. Each peptide was treated to convert phosphoserine to S-

ethylcysteine; then they were resequenced. The results show that S-ethylcysteine was

present at the positions of Ser^7 and Ser^{18} but not at the positions of Ser^9 and Ser^{12}. Mass

spectrographic analysis of the uncleaved, phosphorylated peptide showed the presence

of products with masses of 3,176; 3,256; 3,336 as expected for the unphosphorylated

peptide and derivatives with one and two phosphates. A synthetic peptide

corresponding to mouse p53 residues Met^4-Pro^{13} (Table 1) (SEQ ID NO: 3), and

containing only Ser^7, was phosphorylated very well by DNA-PK. These examples

show that both serine-glutamine (Ser-Gln) sites at the amino terminus of murine p53 are

phosphorylated by DNA-PK. These mouse p53 sites are also phosphorylated *in vivo*,

suggesting that they may be representative of the *in vivo* physiological targets for DNA-

PK.

Similarly, a synthetic peptide corresponding to mouse p53 residues Pro^{16}-Pro^{31},

(SEQ ID NO: 63), Pro Leu Ser Gln Glu Thr Phe Ser Gly Leu Trp Lys Leu Leu Pro

Pro-Lys Lys, is expected to be an effective substrate for DNA-PK, with Ser^{18} being

phosphorylated. In accord with the results with the (SEQ ID NO: 11) variant of the

human p53 amino terminus (see Example 7), a variant of the murine p53 amino

terminus Pro^{16}- Pro^{31}, in which Thr^{21} and Ser^{23} are replaced with alanine residues, (SEQ

ID NO: 64), Pro Leu Ser Gln Glu Ala Phe Ala Gly Leu Trp Lys Leu Leu Pro Pro-Lys

Lys is also expected to be an effective substrate for DNA-PK.

Example 7

Structural requirements for peptide substrates of DNA-PK

To determine the characteristics of peptides derived from the amino-terminal

region of human p53 protein that are specific DNA-PK substrates with excellent kinetic

properties, several series of peptides based on the sequence surrounding serine 15 of

human p53 sequence were made. Each peptide was then tested for substrate activity

(Table 1).

Starting with (SEQ ID NO: 8), Glu^{11}-$[Ser^{15}]$-Lys^{24}-Lys, as a basis for

comparison, individual residues were changed. First, Thr^{18} and Ser^{20} were changed to

alanine, leaving Ser^{15} as the only phosphorylation site (SEQ ID NO: 11) (Table 1).

These changes produced only a twofold increase in K_m and no significant change in

V_{max}. Thus, (SEQ ID NO: 11), which has only one possible site of phosphorylation

corresponding to that for DNA-PK, is as good a substrate as was (SEQ ID NO: 8)

corresponding to the wild-type human p53 sequence, which has another serine and a

threonine residue which may be phosphorylated by other protein kinases. Therefore,

this sequence was chosen as a new base for further sequence changes. Changing Gln^{16}

to glutamic acid (SEQ ID NO: 32) effectively eliminated the ability of the sequence to

act as a DNA-PK substrate. Changing Gln^{16} to asparagine (SEQ ID NO: 33) also

eliminated the ability of the sequence to act as a DNA-PK substrate. This result

indicated that glutamine could not be replaced with either glutamic acid or with a different basic amino acid in DNA-PK substrates. Taken with the above, these results indicated that an adjacent glutamine was required for phosphorylation of serine 15 of human p53-derived amino-terminus peptides.

To determine whether the position of glutamine is critical for phosphorylation activity, first the positions of Gln^{16} and Glu^{17} were switched to form peptide (SEQ ID NO: 20). (SEQ ID NO: 20) had negligible substrate activity, indicating that the glutamine must be immediately adjacent to the phosphate-accepting amino acid. Next, the position of the glutamine was switched to the amino terminal side of the phosphate-accepting amino acid rather than the carboxyl terminal side to form peptide (SEQ ID NO: 14). (SEQ ID NO: 14), the peptide with the positions of Gln^{16} and Leu^{14} switched, was at least as good a substrate as the base peptide (SEQ ID NO: 11) and almost as good a substrate as (SEQ ID NO: 8). Taken with the above results, these results suggested that glutamine must be immediately adjacent to the phosphorylated residue, but it may either follow or precede that residue, *i.e.*, the results indicate that the phosphorylation acceptor amino acid pair in a DNA-PK substrate peptide could be either serine-glutamine (Ser-Gln) or glutamine-serine (Gln-Ser). (SEQ ID NO: 16), with threonine substituted at residue 15, was a slightly better substrate than the base sequence (SEQ ID NO: 11). This result suggests that DNA-PK does not discriminate against threonine sites in these substrates and that threonine-glutamine (Thr-Gln) and glutamine-threonine (Gln-Thr) are acceptable phosphate accepting amino acids pairs in DNA-PK peptide substrates.

(SEQ ID NO: 15), with Leu^{14} changed to glutamine, i.e., where the serine was

surrounded by glutamines, was slightly better as a substrate. This suggests that in

addition to having a phosphate accepting amino acid pair selected from the group

consisting of Ser-Gln, Gln-Ser, Thr-Gln and Gln-Thr, an additional characteristic of the

peptides that are effective substrates for phosphorylation of serine 15 of human p53 by

DNA-PK is the presense of a glutamic acid or glutamine immediately adjacent to the

amino acid pair on either the amino- or carboxyl-side. This is demonstrated by

comparing the phosphorylation of peptides (SEQ ID NO: 13) and (SEQ ID NO: 11) and

by comparing the phosphorylation of peptides (SEQ ID NOs: 15, 14, 11, 18 and 19).

The presence of the adjacent glutamic acid or glutamine residue(s) enhanced

phosphorylation of the peptides, particularly in the cases of peptides (SEQ ID NOS: 15,

18 and 19). Thus, in addition to the phosphate accepting amino acid pair, effective

substrates for DNA-PK also include an enhancer amino acid, which may be either

glutamine or glutamic acid, located immediately adjacent to the amino acid pair on

either the amino- or the carboxyl side of the amino acid pair and forming a phosphate

accepting amino acid pair-enhancer unit.

(SEQ ID NO: 13) has lysine substituted for Glu^{17}, but it has an intact Ser-Gln -

sequence. (SEQ ID NO: 13) is a comparatively poor DNA-PK substrate. This result

suggested that basic residues, other than glutamine, (see results with (SEQ ID NO: 15))

near a Ser-Gln sequence may inhibit recognition and phosphorylation by DNA-PK and

further suggested that, as described above, the glutamic acid that was at this position

may have enhanced recognition and phosphorylation by DNA-PK. In (SEQ ID NO:

17), the leucine (Leu^{14}) is replaced with aspartic acid, making this peptide a less

effective substrate for DNA-PK than peptides (SEQ ID NOS: 15 AND 16). This

suggested that acid residues (other than glutamic acid, see results with (SEQ ID NOS:

18 and 19)), near the Ser-Gln amino acid pair may inhibit phosphorylation by DNA-PK

and further suggested that glutamic acid near the pair acts to enhance phosphorylation

by DNA-PK.

Two additional series of peptides were designed and synthesized and tested to

determine the requisite or desirable lengths of amino acid spacer sequences to be added

on the amino and carboxyl sides of the phosphate accepting amino acid pair and its

attendant glutamic acid or glutamine enhancer (the amino acid pair-enhancer unit) so as

to generate a peptide substrate with excellent kinetic properties while providing a

specific peptide substrate that was additionally cost-effective to synthesize. The first

series was designed to determine the optimum length characteristics for the spacer

sequence on the amino terminal side of the amino acid pair-enhancer unit. This series

is embodied in peptides (SEQ ID NOs: 4, 11, 15, 18 and 19) which have 14, 4, 3, 2

and 1 amino acids, respectively, on the amino-terminal side of the Ser-Gln-Glu, Gln-

Ser-Gln or Glu-Ser-Gln phosphate accepting amino acid pair-enhancer unit. The results

(Table 1) demonstrate that a shorter sequence on the amino terminal side of the

phosphate accepting amino acid pair-enhancer unit enhanced phosphorylation by DNA-

PK. A steady improvement in the kinetic properties of the peptide substrates was

observed as the amino-terminal spacer sequence was shortened. The results suggest

that while peptides with a four-amino acid spacer on the amino terminal side of the

amion acid pair-enhancer unit are effective substrates for DNA-PK, a peptide with a

single amino acid spacer on the amino terminal side of the phosphate accepting amino

acid pair-enhancer unit is an optimal configuration for a DNA-PK substrate and also provides a peptide that is less expensive to synthesize.

The second series of peptides was designed to determine the optimum characteristics for a spacer sequence on the carboxyl terminal side of the amino acid pair-enhancer unit. This series is embodied in peptides (SEQ ID NOS: 8, 11, 10 and 9) which have 7, 7, 4 and 3 amino acids, respectively, on the carboxyl-terminal side of the Ser-Gln-Glu phosphate accepting amino acid pair-enhancer unit. The peptides (SEQ ID NOS: 9 and 10) (Table 1) were phosphorylated at approximately 10% of the rate of the control (SEQ ID NO: 8) by DNA-PK, primarily because of a large increase in K_m. They were also less than 20% as effective substrates as (SEQ ID NO: 11). The results suggest a minimum length for enhanced substrate activity is spacer sequence of seven amino acids on the carboxyl side of the phosphate accepting amino acid pair-enhancer unit. Although (SEQ ID NOs: 3 and 7) are sequences unrelated to serine 15 of human p53 protein, the phosphorylation results with peptides (SEQ ID NOs: 3 and 7), both of which have carboxyl spacer sequences of eight amino acids, suggest that peptides with even longer carboxyl spacer sequences are effective substrates for DNA-PK.

Thus, while the spacer at the amino side of the amino acid pair-enhancer unit may be reduced in length to provide a more effective substrate for DNA-PK and to provide a substrate that is inexpensive to synthesize, the spacer at the carboxyl side of the amino acid pair-enhancer unit cannot be reduced to a length that is substantially less than seven, and when cost of the peptide is not of significance, the carboxyl side spacer sequence could be longer than seven amino acids and the peptide would remain an effective DNA-PK substrate. For example, (SEQ ID NO: 2), which contains two

serines that are phosphorylated by DNA-PK, effectively provides a peptide substrate with two separate carboxyl spacer sequences. One carboxyl spacer, for Glu^6-Ser^7-Gln^8, is twenty-three (23) amino acids in length and the other, for Ser^{18}-Gln^{19}-Glu^{20} is eleven (11) amino acids in length.

As a control to ensure that the substrates which were ineffective DNA-PK substrates had not appeared to be so due to the presence of inhibitory substances in those preparations, all of the poorer substrate peptides, *i.e.*, (SEQ ID NOS: 5, 28, 29, 31, 9, 10, 32, 33, 20 and 13), *i.e.*, were added to (SEQ ID NO: 8) phosphorylation assays at concentrations of up to 250 μM with the concentration of (SEQ ID NO: 8) maintained at 100 μM. None of these peptides significantly inhibited the phosphorylation of (SEQ ID NO: 8). Therefore, these peptides were not ineffective substrates due to the presence of inhibitors of DNA-PK activity that prevented these peptides from being phosphorylated.

Example 8

A second determinant for DNA-PK activation is substrate and kinase binding to DNA

DNA-PK is activated by relatively low concentrations of DNA, and, as was shown by using calf thymus DNA and hsp90, DNA-PK remains active at DNA concentrations well above the K_m for activation (Lees-Miller, et al., 1990, *Mol. Cell. Biol.*, 10:6472-6481). This result was confirmed by using p53 synthetic peptides as DNA-PK substrates and several synthetic oligonucleotides made of alternating dG and dC. The rate of phosphorylation of a synthetic peptide (SEQ ID NO: 11) (Table 1) was

constant at oligonucleotide concentrations of between 5 and >200 μg/ml for dG-dC

oligomers of chain length 16 or above, and this rate was the same as that obtained with

calf thymus DNA.

To determine whether DNA binding affected p53 phosphorylation by DNA-PK,

the rate of phosphorylation of recombinant wild-type mouse p53 was measured as a

function of DNA concentration. At low calf thymus DNA concentrations (0 to

approximately 10 μg/ml), the initial rate of p53 phosphorylation increased sharply but

then decreased as the DNA concentration was further increased. At DNA

concentrations of 100 μg/ml, the rate of p53 phosphorylation fell to approximately 25%

of the rate at 5 μg/ml. A similar dependence of phosphorylation rate on calf thymus

DNA concentration was observed using SV40 TAg or the human Oct-1 POU domain as

substrates.

In contrast to using natural DNA activators, the rate at which wild-type mouse

p53 was phosphorylated when short oligonucleotides were used to activate DNA-PK

was substantially lower at all oligonucleotide concentrations (0 to 100 μg/ml) assayed,

and the rate remained constant at oligonucleotide concentrations above those required

for maximal DNA-PK activation (about 5 μg/ml). Thus, when DNA-PK was activated

by short oligonucleotides, p53 behaved as a substrate very much like hsp90, a non-

DNA-binding substrate. However, when longer calf thymus DNA fragments were used

to activate the kinase, the rate of p53 phosphorylation was highly dependent on the

concentration of DNA at low DNA concentrations, and at high calf thymus DNA

concentrations (above 100 μg/ml), it fell and approached the rate of phosphorylation

obtained with short oligonucleotides. Because it is likely that at low concentrations of DNA, the DNA-binding DNA-PK protein substrates and the DNA-PK are bound to the same DNA molecule and that at high concentrations they are more likely to be bound to different DNA molecules, these results strongly suggest that the rate of substrate phosphorylation may be enhanced substantially when substrate and kinase bind to the same activator DNA fragment. Thus, DNA binding is another factor that influences substrate recognition by DNA-PK.

Example 9

Peptide Glu Pro Pro Leu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys-Lys (SEQ ID NO:11) is a specific DNA-PK substrate

All proteins known to be phosphorylated by DNA-PK *in vitro* are also *in vitro* substrates for other protein kinases. Inspection of the phosphorylation site consensus sequence motifs for other characterized protein kinases reported by Pearson, et al. in *Methods Enzymol.*, 200:62-81 (1991), suggested that none were likely to phosphorylate (SEQ ID NO: 11). This suggestion was tested directly for casein kinase I, casein kinase II, and the catalytic subunit of cyclic AMP kinase. The incorporation of phosphate into (SEQ ID NOS: 11 and 20) by these kinases was less than 5 percent of the incorporation into the standard peptides used to assay each kinase, when assayed at the same concentration (200 or 500 μM). Thus these other kinases did not phosphorylate the DNA-PK peptide substrate (SEQ ID NO: 11) or the DNA-PK negative control peptide (SEQ ID NO: 20) at significant rates compared with their usual substrates. In addition, DNA-PK also did not phosphorylate the casein kinase II substrate peptides Arg Arg Arg Glu Glu Glu Thr Glu Glu Glu (SEQ ID NO: 37) and Arg Arg Arg Asp Asp Asp Ser Asp

Asp Asp (SEQ ID NO: 39), the casein kinase I substrate peptide Asp Asp Asp Glu Glu

Ser Ile Thr Arg Arg (SEQ ID NO: 35), or the S6 kinase substrate Arg Arg Leu Ser Ser

Leu Arg Ala (SEQ ID NO: 36). Less than 0.1 pmole PO_4/min/µg of DNA-PK was

incorporated into each peptide by DNA-PK in 10 min at 30°C when assayed at 200µM.

The results with (SEQ ID NO: 11) are indicative that derivatives of that sequence

are also specific DNA-PK substrates. For example, in (SEQ ID NO: 12) Trp^{23} of (SEQ

ID NO: 11) is replaced by a Leu, which is not a phosphate-accepting amino acid in

kinase reactions. Likewise, (SEQ ID NO: 14), which has the same amino acid

composition as (SEQ ID NO: 11) except that Gln^{16} and Leu^{14} are exchanged to generate

the phosphorylation site Gln^{14}-Ser^{15}-Leu^{16}, is expected to be a specific substrate for

DNA-PK. (SEQ ID NO: 15), in which Leu^{14} is replaced by a Gln residue, is also

expected to be a DNA-PK-specific substrate since the Gln residue cannot accept

phosphate in a kinase reaction. The peptides (SEQ ID NOS: 18 and 19) are also expected

to be DNA-PK specific substrates since they are formed by truncation and rearrangement

of the amino acids comprising the first spacer sequence located at the amino side of the

phosphate-accepting amino acid pair-enhancer unit of the substrate peptide (SEQ ID NO:

11).

Example 10

(SEQ ID NO: 11) specifically detects DNA-PK activity in crude cell extracts

(SEQ ID NO: 11) (Ser-Gln-Glu peptide) and (SEQ ID NO: 20) (Ser-Glu-Gln

peptide), as shown in Table 1, were examined to determine whether they would function

as specific indicators of the presence of DNA-PK activity in crude cell extracts. To

examine cells for DNA-PK content, extracts from HeLa, T98G human glioblastoma, and

mouse L 929 cells were prepared by freeze-thaw lysis of cells harvested from five nearly

confluent 9-cm-diameter plates as described in Example 1 and by Lees-Miller, et al. *J.*

Biol. Chem., 264:2431-2437 (1989). T98G (ATCC CRL 1690) is a human glioblastoma

cell line; L-929 (ATCC CCL 1) was derived from connective tissue of the NCTC 2071

mouse. Based on earlier work with natural protein substrates, it was expected that

extracts of HeLa and T98G cells would contain a significant amount of DNA-PK activity

while the extracts of mouse L 929 would contain little, if any, DNA-PK activity. Lysates

in low-salt buffer were centrifuged at 10,000 x g, to give a supernatant fraction

(designated S10) and pellets (designated P10). The pellets were washed by resuspension

in a high salt buffer (0.5 M KCl, 25 mM N-2-hydroxyethylpiperazine-N'-2-

ethanesulfonic acid (HEPES; pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol) and were

sonicated. The sonicated suspension was centrifuged at 10,000 x g for 10 min to give a

second supernatant fraction (designated P10S), which was diluted with buffer lacking

KCl to give a volume equal to that of the original supernatant (S10). Protein

concentrations, determined using the Bio-Rad dye-binding assay, were as follows: HeLa

S10, 3 mg/ml; HeLa P10S, 1 mg/ml; T98G S10, 3.5 mg/ml; T98G P10S, 1 mg/ml; L-929

S10, 1 mg/ml; and L-929 P10S, 0.3 mg/ml. These extracts were assayed for the ability to

phosphorylate (SEQ ID NOS: 11 and 20) with and without added calf thymus DNA. The

results are shown in Table 2.

Table 2

Phosphorylation of synthetic peptides in crude cell extracts

Cells and Fraction[a]	(SEQ ID NO: 11) Ser-Gln-Glu		(SEQ ID NO: 20) Ser-Glu-Gln	
	PO$_4$ incorporated (nmol/min/ml of extract)[b]			
	+ DNA	- DNA	+ DNA	- DNA
HeLa				
S10	33.8	0.8	0.3	0.0
P10S	28.9	6.5	0.0	0.0
T98G				
S10	22.6	0.2	0.04	0.02
P10S	15.0	1.2	0.0	0.0
L 929				
S10	0.0	0.0	0.0	0.0
P10S	0.005	0.0	0.0	0.0

[a]The soluble fraction (S10) and a salt wash (P10S) of the insoluble fraction, in equal volumes, were prepared from about 2.5 X 10^7 cells as described.

[b]Extracts were incubated for 10 min at 30° C with the indicated peptide (Table 1) at 200 µM and with (+) or without (-) calf thymus DNA at 10 µg/ml. Phosphate incorporation was approximately linear for the assay period (data not shown). Background values from reactions without peptide were substracted.

As shown in Table 2, extracts of the two human cell lines phosphorylated (SEQ ID NO: 11) at rates that were 200-300 times that of the assay background. Furthermore, phosphorylation by the S10 fractions was entirely dependent on added DNA. Phosphorylation by the P10S fraction was partially dependent on added DNA. This suggests that some DNA was present in the sonicated, salt wash of the freeze-thaw 10,000g pellet, which is expected since some DNA of the nuclei of the pellet would be extracted by sonication in high salt. In contrast, (SEQ ID NO: 20) was poorly phosphorylated, if at all, by all extracts. The level of phosphate incorporated into (SEQ ID NO: 20) was not significantly different from the amount of background radioactivity on the filter due to residual radioactivity from the ATP preparation. Table 2 also shows that DNA-dependent kinase activity was distributed about equally between the S10 and

P10S fractions. Western immunoblot analysis, using an antibody against a partial DNA-PK cDNA fusion protein (anti-DNA-PK antibody), confirmed that roughly equal amounts of the 350-kDa DNA-PK peptide were present in the two fractions.

Mouse L 929 cell extracts did not appear to contain DNA-PK. (SEQ ID NO: 11) was phosphorylated at a rate that was at least 100 times lower than the rates at which it was phosphorylated by the human cell extracts. This result is consistent with the previous observation that mouse L 929 cell extracts did not phosphorylate hsp90 in a DNA-dependent manner. L 929 cell extracts also lacked a 350-kDa polypeptide that reacted with the anti-DNA-PK antibody. These results indicate that mouse L 929 cells have little DNA-PK activity compared with the cultured human cells that have been examined. Several other rodent cell lines, including SV40-transformed BALB/c mouse embryo fibroblasts and secondary rat embryo fibroblasts, also had little if any DNA-PK activity. Rodent cell extracts are well-known to have other kinases, including casein kinases I and II. Therefore, these results also demonstrate that (SEQ ID NOS: 11 and 20) are not effective substrates for the common kinases of mammalian cells. Thus, (SEQ ID NO: 11) represents a specific peptide substrate for detection and quantitation of DNA-PK activity in cell extracts and other biological samples. It is expected that the previously discussed derivatives of (SEQ ID NO: 11), (SEQ ID NOS: 12, 14, 15, 18 and 19), are also specific peptide substrates for detection and quantitation of DNA-PK activity in celll extracts and other biological samples.

Example 11

Cultured somatic cells from primates have high concentrations of DNA-PK compared to cultured somatic cells from other eukaryotes

Extracts of additional primate and rodent cells were tested for the presence of DNA-PK activity in accordance with the method of the present invention. The DNA-PK activity was determined using the methods described in Examples 1, 4 and 5 for seven human cell lines, two African green monkey kidney lines, and six rodent cell lines. These results are shown in Table 3.

Extracts were prepared essentially as described in Example 10. Cells were lysed by freezing and thawing in hypotonic buffer; soluble (S10) "cytoplasmic" and insoluble (P10) "nuclear" fractions were made by centrifuging lysates at 10,000 x g for 10 min. The insoluble fraction was extracted with buffer containing 0.5 M KCl, which releases any DNA-PK that is bound to DNA. After centrifugation of the salt-washed pellet fraction, which removed the remaining insoluble material, the salt extract (P10S) was diluted and assayed as previously described.

Protein concentrations, determined using the Bio-Rad dye binding assay, were between 0.3 and 6 mg/ml. Extracts were incubated at 30°C for 10 min with each peptide at 0.2 mM in the absence of added DNA (-DNA) or with 10 µg/ml calf thymus DNA (+DNA). After incubation the reaction mixtures were acidified with an equal volume of 30% acetic acid and spotted on to Whatman P-81 phosphocellulose paper (Whatman, Inc., Clifton, NJ). The filters then were washed extensively in 15% acetic acid before the adsorbed radioactivity was quantitated as described in Example 5.

The amount of phosphate incorporated was calculated from the specific activity of the ATP. The rate of phosphate incorporation into the Ser-Gln-Glu peptide (Table 1) (SEQ ID NO: 11) in the presence of calf thymus DNA was approximately linear throughout the 10 minute reaction incubation period. Parallel assays were performed without added peptide, and these background values were subtracted to produce the activities that are shown in Table 3. Background values varied but, in all cases, were less than 0.2 nmol/min/ml (a few percent of the activity present in human cells). The specific activities were calculated from the total activity in the two fractions divided by the total protein in the two fractions. The percent of total DNA-PK activity in the S10 and P10S fractions was determined with peptide (SEQ ID NO: 11) in the presence of added calf thymus DNA. Extracts without substantial DNA-PK activity were examined for their ability to inhibit purified DNA-PK. Inhibitory activity was not detected.

As shown in Table 3, the extracts of human cells lines have DNA-PK activity that is readily detectable with the Ser-Gln-Glu (SEQ ID NO: 11) peptide, and in each case phosphorylation of the Ser-Gln-Glu peptide by supernatant fractions was highly dependent on added calf thymus DNA (Table 3). DNA-dependent phosphorylation of the Ser-Gln-Glu peptide also was observed with S10 extracts from CV-1 and COS-1 African green monkey cells, but little phosphorylation of the Ser-Gln-Glu peptide was observed with extracts from any of the six rodent cell lines. In addition, little, if any, phosphorylation of the Ser-Gln-Glu peptide was seen using extracts of *Drosophila* Kc cells or in *Spodoptera fragiperda* (fall army worm) Sf9 cells. The Ser-Gln-Glu peptide was also phosphorylated by the salt wash of the insoluble fraction (Table 3, P10S) from human and monkey cells, but substantial phosphorylation by these fractions occurred

without added DNA. The apparent DNA-independent phosphorylation observed with P10S fractions probably results from the presence of endogenous chromatin-derived DNA fragments, as previously discussed in Example 10. None of the extracts phosphorylated the Ser-Glu-Gln peptide (SEQ ID NO: 20) at a significant rate, with or without added DNA.

Table 3

DNA-PK Activity in Extracts of Primate and Rodent Cells

Species & Cell Line	Fraction	Specific Activity (nmol PO4/min/mg protein)					
		(SEQ ID NO: 11)				(SEQ ID NO: 20)	
		Specific Activity		% of Total	Total	Specific Activity	
		+ DNA	- DNA			+ DNA	- DNA
Human MRC 5[a]	S10	13.6	0.01	97.9		0.30	0.11
	P10S	3.1	1.02	2.1	15.1	0.04	0.02
Human HeLa-JW[b]	S10	11.0	0.26	72.5		0.1	0.0
	P10S	24.9	5.6	27.5	14.9	0.1	0.0
Human T98G[c]	S10	6.46	0.06	60.6		0.01	0.01
	P10S	12.9	1.0	39.4	7.4	0.0	0.0
Human HPB-ALL[d]	S10	4.5	0.05	28.5		0.0	0.04
	P10S	22.1	14.7	71.5	10.4	0.05	0.04
Human 293-S[e]	S10	3.1	0.3	34.5		0.01	0.0
	P10S	16.3	0.4	64.5	6.4	0.04	0.0
Human A549[f]	S10	7.5	0.1	28.6		0.0	0.02
	P10S	31.1	0.9	71.4	16.4	0.01	0.06
Human Raji[g]	S10	3.8	0.6	47.9		0.0	0.08
	P10S	13.5	15.5	52.1	6.0	0.0	0.0
Monkey CV-1[h]	S10	5.6	0.8	42.0		0.05	0.0
	P10S	18.1	6.3	58.0	9.4	0.08	0.08
Monkey COS-1[i]	S10	3.8	0.85	54.5		0.02	0.0
	P10S	10.7	8.4	45.5	5.4	0.24	0.0
Mouse L-929[j]	S10	0.0	0.0	--		0.0	0.0
	P10S	0.02	0.0	--	0.0	0.0	0.0
Mouse Balb/c MEF	S10	0.10	0.01	--		ND[o]	ND
	P10S	0.15	0.09	--	0.1	ND	ND
Mouse SV-Balb/c[k]	S10	0.04	0.01	42		0.06	0.0
	P10S	0.20	0.15	58	0.1	0.04	0.0
Rat REF[l]	S10	0.05	0.03	8		0.0	0.02
	P10S	0.50	0.38	92	0.3	0.0	0.03
Hamster V79[m]	S10	0.11	0.01	39		0.0	0.0
	P10S	0.61	0.0	61	0.2	0.05	0.05
Hamster CHO[n]	S10	0.06	0.05	32		0.01	0.0
	P10S	0.05	0.36	68	0.2	0.06	0.0

[a]MRC-5 (ATCC CCL 171) cells are secondary diploid cells from normal human lung tissue (obtained from J.C. Barrett, NIEHS, Research Triangle Park, NC).

[b]HeLa-JW are a derivative of HeLa cells (ATCC CCL 2) selected for use in adenovirus plaque assays (obtained from J. Williams, Carnegie-Mellon University, Pittsburg, PA).

[c]T98G cells (ATCC CRL 1690) are derived from a human glioblastoma (obtained from E. Appella, Laboratory of Cell Biology, Building 37, NIH Bethesda, MD).

[d]HPB-ALL are a human T-cell derivative (obtained from D. Morrisson, Frederick Cancer Research Center, Frederick, MD).

[e]293-S (from ATCC CRL 1573) are suspension adapted primary human embryonic kidney cells transformed by adenovirus 5 DNA (obtained from B. Stillman, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY).

[f]A549 (ATCC CCL 185) was derived from human lung carcinoma tissue (American Type Culture Collection, Rockville, MD).

[g]Raji (ATCC CCL 86) are lymphoblast-like cells derived from a Burkett lymphoma (American Type Culture Collection, Rockville, MD).

[h]CV-1 cells (ATCC CCL 70) are immortalized African Green Monkey kidney cells (American Type Culture Collection, Rockville, MD).

[i]COS-1 (ATCC CRL 1650) were derived from CV-1 cells by transformation with origin defective SV40 DNA (American Type Culture Collection, Rockville, MD).

[j]L-929 (ATCC CCL 1) was derived from connective tissue of the NCTC 2071 mouse (obtained from the Tissue Culture Facility of the State University of New York at Stony Brook, Stony Brook, NY).

[k]SV-Balb/c are early-passage SV40-transformed primary Balb/c mouse embryo cells (obtained from A. Lewis, Viral Pathogenesis Section, Building 7, NIH, Bethesda, MD).

[l]REF were early-passage secondary rat embryo cells (obtained from P. Tegtmeyer, State University of New York at Stony Brook, Stony Brook, NY).

[m,n]V79 and CHO (ATCC CCL-61) are Chinese hamster cell lines derived from lung and ovary tissue, respectively (obtained from M. Bender, Medical Department, Brookhaven National Laboratory, Upton, NY).

[o]ND = Not determined.

Example 12

Optimal DNA-PK assay conditions

Experiments were performed to determine optimal DNA-PK assay conditions. It was determined that DNA-PK is active over a broad pH range and in most common buffers with the expected exception of phosphate buffers. A summary of assay conditions that optimize DNA-PK activity *in vitro* is found in Table 4 along with a summary of conditions that result in 50% inhibition of DNA-PK activity.

As shown in Table 4 the influence of ionic strength on activity is modest at concentrations below physiological concentrations. Activity decreases significantly at higher salt concentrations, presumably because the enzyme can no longer bind to DNA. Activity requires a divalent cation, magnesium being more effective than manganese. Calcium and zinc are poor substitutes for magnesium. DNA-PK activity was inhibited by phosphate, pyrophosphate, heparin, and by high concentrations of glycerol. Purified

DNA-PK is active in 0.5% NP-40 or Triton-X100, but little DNA-dependent

phosphorylation is observed in cell lysates prepared with these non-ionic detergents.

Table 4

Assay Conditions for DNA-PK

Parameter	Normal Reaction Concentration	Effective Range
pH	7.5	6.8 – 9.2
Salt (mM)	100	20-120
$MgCl_2$ (mM)	10.0	$\approx 1 - > 10$
ds DNA (µg/ml)	10.0	$\approx 2 - > 1000$
ATP (mM)	0.5	$0.02 - > 5$
Specific Activity (µCi/µmol)	300	
Substrate Peptide (mM)	200	$\approx 50 - \approx 1000$
Protein (mg/ml)	0.1	$\approx 0.01 - \approx 1$

Inhibitor	50% Inhibition
Salt (mM)	180
Glycerol (%v/v)	25
Phosphate (mM)	10
Pyrophosphate (mM)	3
Heparin (µg/ml)	1
Single Stranded DNA (µg/ml)	20

The methods used to obtain results in the second section (Inhibitor section) of

Table 4 are illustrative of methods that are applicable to identifying substances that alter

the activity of DNA-PK. In each assay, the concentrations of the suspected inhibitory

substances was increased and the results compared to reaction assays that did not contain

the substances, particularly in the cases of Glycerol, phosphate, pyrophosphate, heparin

and single stranded DNA. Comparable assays could be used to detect and assess

substances that altered DNA-PK activity in a positive manner to stimulate DNA-PK

activity.

Furthermore, as in Example 11, cell extracts can also be examined for the

presence of substances that alter the activity of DNA-PK. For example, the Rat REF cell

extracts displayed very little DNA-PK activity. If these cell extracts appeared negative

for DNA-PK because of the presence of an inhibitory substance or compound, if such extracts were added to standard *in vitro* DNA-PK assays in a manner illustrated by the second section of Table 4, DNA-PK activity would have been reduced as the amount of extract added to the reaction was increased. As this was not found, the extracts did not contain substances that negatively affected DNA-PK activity. Conversely, cell extracts containing substances and compounds that enhance DNA-PK activity could be identified by similar procedures.

Example 13

Isolation of phosphopeptides by immobilized metal chromatography

Immobilized metal affinity chromatography provides a method for separating phosphopeptides from similar or identical non-phosphorylated peptides. Phosphate binds tightly to chelated iron at acid pH (Muszyhska, et al., 1986, *Biochemistry*, 25:6850-6853; Anderson, et al., 1986, *Anal. Biochem.*, 154:250-254). Most non-phosphorylated peptides and proteins do not bind immobilized metal ions. The ability to purify phosphopeptides is important for identifying sites in proteins that are phosphorylated. This technique could be applied to a method for detecting DNA-PK activity in the absence of a radioactive phosphate donor.

Phosphopeptides are isolated using iminodiacetic acid (chelating)-Sepharose 6B (Sigma Chemical Co., St. Louis, MO). This procedure is used for 1-5 mg of peptides. This size column is appropriate for ~200 nmoles of phosphoprotein. All steps are performed at room temperature unless otherwise indicated.

A 1 x 6 cm (5 ml capacity) column of chelating-Sepharose 6B (Pharmacia-LKB Biotechnology, Piscataway, NJ or Sigma, St. Louis, MO) or an equivalent IDA-resin

(Sigma Chemical Co., St. Louis, MO) is washed with 4 volumes (20 ml) of H_2O. The theoretical capacity is about 2 micromoles of phosphoprotein. The column is then washed with 4 volumes (20 ml) of 50 mM Tris-HC1, pH 7.6, 100 mM EDTA, and 500 mM NaC1, followed by a wash with 4 volumes (20 ml) of H_2O. The column is then saturated with iron by washing with 4 volumes 50 mM ferric chloride. After saturation the column is washed with 2 volumes 0.1 M acetic acid to remove any loosely bound iron. The peptide mixture, produced from a phosphorylation reaction as described in Example 4, is loaded onto the column in 4 ml of 0.1 M acetic acid. After loading, the column is washed with 4 volumes (20 ml) of 0.1 M acetic acid to remove any unbound peptides. The column is then washed with 4 volumes (20 ml) of 0.1 M sodium acetate, pH 5.0 (0.1 M acetic acid adjusted to pH 5.0 with sodium hydroxide). Finally, the column is washed with 4 volumes (20 ml) of 1% ammonium acetate (pH 6.3).

Phosphopeptides are eluted with 4 column volumes 1% ammonium acetate adjusted to pH 8.3. Samples are collected in 0.5-1.0 ml sample volumes and pooled according to pH (spot test on paper). Samples are concentrated by Speed-vac (Savant Instruments, Farmingdale, NY). The sample may be quantitated, for example, by amino acid analysis or by UV absorption to determine how much phosphopeptide was obtained.

Example 14

Measurement of chemical phosphate by Malachite-Green assay

An additional method to perform protein kinase assays without using radio-labeled phosphate donors makes use of a Malachite-Green assay for chemical phosphate. The following procedure is applicable to detection and quantitation of DNA-PK activity using natural protein substrates or using synthetic peptide substrates.

After performing phosphorylation reactions as described in the Example 4, the measurement of chemical phosphate in protein is performed using the Malachite-Green assay. The Malachite-Green assay is used exactly as described by Buss and Stull in *Methods Enzymol.*, 99:7-14 (1983) as follows. The useful range of the assay is 0.2 to 1.5 nmoles of protein phosphate. Accurate measurements require duplicate or triplicate samples and a similar quantity for measurement of protein content. Protein concentration is best determined by duplicate amino acid analysis unless an accurate extinction coefficient is known. Common problems with this assay include: (1) high backgrounds from phosphate contaminated reagents or glassware, (2) adherence of non-covalently associated protein phosphate, (3) incomplete precipitation of protein by TCA and (4) inaccurate measurement of total protein. The precautions described by Buss and Stull are followed scrupulously, and positive and negative controls are included with each assay. The assay for samples and for the standard curve are processed in exactly the same way. A sample standard curve is shown in Table 5.

To measure DNA-PK activity using this assay it is essential to first remove the ATP and any inorganic phosphate from the sample. With protein substrates such as hsp90, this can be accomplished by TCA precipitation. Many short peptides are soluble in TCA. Thus, the peptide must first be purified from ATP and any non-peptide bound phosphate using properties of the peptides including the tag moiety. These might include gel filtration as described in Example 5, purification on and elution from phosphocellulose, or purification using a different tag moiety. The purified or partially purified phosphopeptide, free of other sources of phosphate, is redissolved in phosphate-free water or dilute acid, and then added to the assay in a manner similar to

the phosphate solution that is used to create the standard curve, a sample of which is shown in Table 5.

Table 5

Phosphate Standard Curve

No.	Description	PO$_4$ Std. (μl)	1.2N HCL (μl)	pmol P	Expected A660 nm
1	(blank)*	0	300	0	0.02
2	(blank)*	0	300	0	0.02
3	10 μM	5	295	50	0.02
4	10 μM	10	290	100	0.03
5	10 μM	20	280	200	0.04
6	10 μM	30	270	300	0.06
7	20 μM	20	280	400	0.08
8	20 μM	25	275	500	0.10
9	20 μM	30	270	600	0.12
10	20 μM	35	265	700	0.14
11	20 μM	40	260	800	0.16
12	20 μM	50	250	1000	0.23
13	20 μM	60	240	1200	0.30
14	20 μM	80	220	1600	0.37
15	20 μM	100	200	2000	0.45

*Two blanks are averaged and subtracted from experimental readings. Samples and controls are assayed using new borosilicate tubes.

Controls are precipitated and washed as above (in duplicate). Negative controls may comprise non-phosphorylated proteins, *e.g.* BSA, and positive controls may comprise phosphorylated proteins such as ovalbumin, which contains approximately 0.6 moles of phosphate per 0.74 moles of protein.

For the assay, 100 μl ashing reagent (10% MgNO$_3$) is added to each TCA precipitate. The samples are warmed in a heating block at 70°C for 10 min. and the the dried yellow pellet is carefully heated over a Bunsen flame until all brown smoke is gone and a white powder remains. The tube is placed in a metal rack to cool and then 300 μl 1.2 N HCl and 100 μl color reagent is added, mixed, transferred to a

microcuvette and the optical density at 660 nm is determined and compared to a

standard curve prepared from phosphate solutions of known concentration as described

above.

Example 15

Construction of DNA-PK substrate vectors for expression in *E. coli*

Construction of DNA-PK Substrate Vector Precursor

The human Oct-1 POU domain gene was obtained in plasmid

pET11cOCT1POU from R. Aurora and W. Herr (Cold Spring Harbor Laboratory) and

moved into a derivative of the Novagen T7 plasmid vector pET-3, pT7HEP1DBP, as

described (Anderson and Lees-Miller, 1992, *Crit. Rev. in Eukaryotic Gene Expression*,

2:283-314). The resulting plasmid was called pT7HPOU1 or p236. The predicted

sequence is shown Figure 5B. A map of which is shown Figure 5A.

A plasmid with a mutation in the POU domain which prevents binding to DNA

was obtained from W. Herr; this plasmid was referred to as p335. In this mutant,

codon 49 of the POU domain was changed from arginine (Arg) to alanine (Ala), and

the mutant POU domain was referred to as the R49A mutant. The R49A mutation also

introduced a *Hae* III site in the gene. R49 (of the POU domain) is at amino acid

position 79 of the nascent, wild-type, DNA-PK recombinant substrate shown in Figure

8B.

A double stranded oligonucleotide segment that encodes the phosphorylation site

segment Met Pro Glu Glu Ser Gln Glu Thr Phe Glu Asp Leu Trp Lys Leu Leu Pro Gly

His His (SEQ ID NO:45) was prepared by annealing BNL oligonucleotides #2459

(forward) and #2458 (reverse) shown in Table 6.

Table 6

#2459 Sense-strand oligonucleotide

5'-TATG<u>CCTGAGG</u>AAAGTCAGGAGACATTCGA<u>AGATCT</u>ATGGAAACTACTTCCTG-3'
      (CC|TGAGG) *Bsu*36 I   (A|GATCT) *Bgl* II
                   (SEQ ID NO: 46)

#2458 Antisense-strand oligonucleotide

5'-GTGACCAGGAAGTAGTTTCCATAGATCTTCGAATGTGTCCTGACTTTCCTCAGGCA-3'
                   (SEQ ID NO: 47)

The annealed double stranded oligonucleotide had a 5' extension at the (SEQ ID NO: 46) 5' end that was compatible with an *Nde* I site (CA|TATG) and the (SEQ ID NO: 47) 5' end had an extension compatible with a *Bst*E II site (G|GTNACC). A *Bsu*36 I site (CC|TGAGG) (at the Pro Glu codons) and a *Bgl* II site (A|GATCT) (at the AspLeu codons) were also present in the oligonucleotides at internal positions. The double stranded oligonucleotide (#2459:#2458) was cloned into *Nde* I and *Bst*E II-cleaved p236 (pT7HPOU1) using standard techniques known in the art (Sambrook, et al., 1989, *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Press, NY). This intermediate construct was not given a name.

A derivative of pET-25b lacking a *Bgl* II site (Novagen) was constructed by cleaving pET-25b DNA with *Bgl* II, followed by treating the linearized plasmid with Klenow polymerase to fill in the site and with T4 Ligase to re-circularize the plasmid. The resulting pET-25b derivative without a *Bgl* II site was called p409. A similar derivative of pET-28a DNA was subsequently prepared in an identical manner and designated p410.

Construction of Substrate Expression Cassettes

The segment encoding the Oct-1/POU sequence in the unnamed intermediate construct was copied using polymerase chain reaction (PCR). The primers were #2486 (5' sense-strand), #2485 (3' antisense strand) and the template DNA was the unnamed intermediate plasmid. The sequences of primers #2486 and 2485 are shown in Table 7.

Table 7

#2486 5' sense primer with upstream untranslated segment and 5'*Xba* I site for substrate PCR

5'-GCTCTAGAAGTCGACTTTAAGAAGGAGATACCAAGATGCCTGAGGAAAGTCAG-3'
(SEQ ID NO: 48)

#2485 3' Antisense primer with HSV epitope sequence and 3' *Bam*H I site for substrate PCR

5'-CGGGATCCTAATCCTCAGGGTCTTCCGGGGCGAGCTCTGGCTGTGGGTTGATTCTTTTTTC-3'
(SEQ ID NO: 49)

The resulting PCR-generated fragment was cleaved with *Xba* I and *Bam*H I and cloned into similarly cleaved p409 (pET-25b-derivative). The resulting plasmid was called p318 or p318SUB1.

Plasmid p318SUB1 has a *Bsu*36 I site and a *Sal* I site near the 3' end of the *Xba* I - *Bam*H I substrate expression cassette; these sites were removed to facilitate subsequent cloning operations. The Oct-1/POU region of p318SUB1 was copied by PCR using primers #2602 and #2601, which are shown in Table 8. The nucleotide changes that removed the *Bsu*36 I and the *Sal* I sites are in the 3' antisense primer #2601.

Table 8

#2602 5' Sense-strand primer for substrate PCR.
5'-CATCA<u>CCATGG</u>TATGAGCGGCGGCATGGAGGAGCCCAGTGACCTTG-3'
       (C|CATGG) *Nco* I

(SEQ ID NO: 50)

#2601 3' Antisense primer for substrate PCR.
5'-CG<u>GGATCC</u>TAATCCTCGGGGTCTTCCGGGGCGAGTTCTGGCTGTGGGTTGATTCTTTTTTC-3'
   BamH I ***Stop x*Bsu*36 I   x*Sal* I
(SEQ ID NO: 51)

The PCR-produced fragment was cleaved with *Nco* I (C|CATGG) and *BamH* I

and cloned into similarly cleaved p318SUB1; the resulting plasmid is called p345 or

p345SUB1. The non-expressing *E. coli* strain DH-5α was transformed with this

plasmid and designated strain CWABNL #345.

A derivative of p345SUB1 was prepared which does not bind DNA. The Oct-

1/POU segment from plasmid p335, which contains the R49A mutation, was copied by

PCR using primers #2601, #2602 and p335 DNA as template. The resulting fragment

was cleaved with *Nco* I and *BamH* I as described above and cloned into similarly

cleaved p318SUB1. This plasmid was designated p346 or p346SUB2. As with

P345SUB1, P346SUB2 was also introduced to the non-expressing *E. coli* strain DH-

5α.

Construction of Expression Vectors p349SUB1 and p350SUB2

The substrate expression cassettes from p345SUB1 and p346SUB2 were moved

from the pET-25 background, which is selectable with the antibiotic ampicillin, to

p410, a derivative of pET-28, which is selectable with 50 μg/ml of the antibiotic

kanamycin. The *Xba* I - *BamH* I substrate cassettes were excised from p345 and p346

and cloned into *Xba* I and *Bam*H I cleaved p410. The resulting plasmids were called

p349SUB1 and p350SUB2, respectively. A map of plasmid p349SUB1 is shown in

Figure 8A. The predicted sequence for p3495SUB1 was designated Wild-Type

Artificial DNA-PK substrate 1 and is shown in Figure 8B.

Construction of Expression Vectors p355SUB3 and p357SUB5

To generate expression vector constructs that expressed negative control

proteins, derivatives of p349SUB1 and p350SUB2 were prepared by replacing the

serine residue codon at the phosphorylation site with the codon for the non-

phosphorylatable residue alanine (Ala). These recombinant negative control protein-

expressing plasmids were constructed by replacing the short segment between the

*Bsu*36 I and the *Bgl* II sites located in the p53 substrate phosphorylation site encoding

segment of the p53 gene with a double stranded oligonucleotide prepared by annealing

oligonucleotides #2738 and #2739. Oligonucleotides #2738 and #2739 are shown in

Table 9. The mutation-containing fragment also has a diagnostic *Bst*N I site

(CC|WGG) which is not present in the wild-type substrate.

Table 9

#2739 Synthetic substrate fragment to change sequence from
Ser Gln Glu to Ala Gln Glu, sense strand.

```
        Glu Glu Ala Gln Glu Thr Phe Glu     (SEQ ID NO: 52)
    5'-T GAG GAA GCC CAG GAG ACA TTC GAA-3' (SEQ ID NO: 53)
```

#2738 Synthetic substrate fragment to change Ser Gln Glu
site to Ala Gln Glu, antisense strand.

```
    5'-GATCTTCGAATGTCTCCTGGGCTTCC-3'   (SEQ ID NO: 54)
```

The resulting plasmids were designated p355SUB3 (wild-type Oct-1/POU

domain) and p357SUB5 (R49A mutant Oct-1/POU domain), respectively.

Construction of Expression Vectors p356SUB4 and p358SUB6

In the same manner as described in the previous section, additional negative control-expressing vectors were prepared. Derivatives of p349SUB1 and p350SUB2 that had the sequence at the DNA-PK recognition site -Ser Gln Glu- changed to -Ser Glu Gln- were constructed. The Ser Glu Gln site in a peptide is not phosphorylated by DNA-PK. A replacement *Bsu*36 I - *Bgl* II fragment was prepared by annealing oligonucleotides *#2740* and *#2741* (Table 10). This fragment also has a diagnostic *Ple* I site (GAGTCNNN|).

<div align="center">Table 10</div>

#2741 Synthetic substrate fragment to change phosphorylation site sequence from the wild-type Ser Gln Glu to Ser Glu Gln, sense strand.

      5'-TGAGGAGTCTGAGCAGACATTCGAA-3' (SEQ ID NO: 55)

#2740 Synthetic substrate fragment to change phosphorylation site sequence from the wild-type Ser Gln Glu to Ser Glu Gln, antisense strand.

      5'-GATCTTCGAATGTCTGCTCAGACTCC-3' (SEQ ID NO: 56)

The resulting plasmids were designated p356SUB4 (wild-type Oct-1/POU domain) and p358SUB6 (R49A mutant domain), respectively.

Confirmation of Recombinant Sequences

The expected nucleotide sequence for each gene encoding an artificial recombinant protein substrate and control protein was confirmed by DNA sequence analysis of the vectors and N-terminal protein sequence analyses of the expressed proteins. This was accomplished by sequencing one strand of the substrate-encoding region of each plasmid and by subjecting each of the purified proteins to automated Edman degradation using an Applied Biosystems 430A protein sequencer.

Expression of Artificial Substrate Encoding Genes

In order for expression of the encoded substrates to occur, each plasmid was transferred to the *E. coli* expression strain BL21(DE3). These transformed strains were designated:

> **#351** = p349SUB1 in BL-21(DE3): Artificial Substrate Expression Vector with Ser Gln Glu DNA-PK site and wild-type p53-Oct1/POU domain.

> **#352** = p350SUB2 in BL-21(DE3): Artificial Substrate Expression Vector with Ser Gln Glu DNA-PK site and R49A mutant p53-Oct1/POU DNA binding domain. POU domain has *Hae* III site that is not present in wild-type DNA.

> **#386** = p355SUB3 in BL-21(DE3): Control DNA-PK Artificial Substrate Expression Vector with Ala Gln Glu (S4A) "DNA-PK site" and wild-type DNA binding domain. p53 segment has *Bst*N I site (CC|WGG) that is not in wild-type DNA.

> **#387** = p356SUB4 in BL-21(DE3): Control DNA-PK Artificial Substrate Expression Vector with Ser Glu Gln "DNA-PK site" and wild-type DNA-binding domain; p53 segment has *Ple* I site that is not in wild-type DNA.

> **#388** = p357SUB5 in BL-21(DE3): Control DNA-PK Substrate Artificial Expression Vector with Ala Gln Glu (S4A) "DNA-PK site" and R49A mutant DNA-binding domain. Plasmid has *Bst*N and *Hae* III sites that are not in wild-type plasmid.

> **#389** = p358SUB6 in BL-21(DE3): Control DNA-PK Artificial Substrate Expression Vector with Ser Glu Gln "DNA-PK site" and R49A mutant DNA-binding domain. Plasmid has *Ple* I and *Hae* III sites that are not in wild-type plasmid.

Each of the above strains produced an approximately 20 kDa polypeptide corresponding to the respective substrate following induction for expression with 1 mM IPTG (isopropyl-[β]-D-thiogalactopyranoside) using methods for cell growth and induction as descibed by Studier, et al. (*Met. Enz.*, 185:60-189, 1990). Each substrate was purified to >90% homogeneity from IPTG-induced *E. coli* cell lysates by Ni-affinity

chromatography using a Pro-bond (Ni) resin (Invitrogen, Cat# R801-01, 3985 B Sorrento

Valley Blvd, San Diego, CA 92121) according to the manufacturer's instructions.

Example 16

Characterization of recombinant (artificial) substrates produced in _E. coli_

The recombinant protein substrates, a total of six, (at approximately 0.1 mg/ml

final concentration) were separately mixed with hsp90 in a phosphorylation reaction

with purified DNA-PK (0.6 μg, 0.02 units/assay) without (-) or with (+) 5 μg/ml calf

thymus DNA, in the presence of ^{32}P-ATP for 30 minutes at 30°C as described by Lees-

Miller, et al., _Mol. Cell. Biol._, 12:5041-5049 (1992). The phosphorylation reactions

were stopped with SDS sample buffer and analyzed with SDS-PAGE. The gel was

fixed, stained with Coomassie brilliant blue, dried and developed by autoradiographic

procedures. The results of which can be seen in Figure 9. The upper panel of Figure 9

shows the phosphoimage (Molecular Dynamics, 880 East Arques Ave Sonnyvale, CA

94086) of the gel, revealing the transfer of ^{32}PO$_4$ to the control protein (hsp90) and to

the recombinant artificial substrate protein (rSub); the two lower panels show

Coomassie blue stain images of regions corresponding to hsp90 and the recombinant

substrates.

As would be apparent to one skilled in the art, Figure 9 shows the recombinant

substrates behaved as expected with respect to phosphorylation by purified DNA-PK.

The wild-type recombinant DNA-PK substrate (1 ESQE-DBP+) was phosphorylated in

a DNA-dependent manner equal to, if not better than, hsp90 (a known DNA-PK

substrate). Furthermore, a 20-fold increase in phosphate incorporation into both

substrates was observed by the addition of double stranded DNA compared to no addition of DNA.

Substrates containing mutant DNA-PK phosphorylation sites with the serine changed to alanine, i.e., substrates 2 and 5, were phosphorylated to less than 5% of the level of the wild-type substrate. Also, substrates containing mutant DNA-PK phosphorylations sites with the glutamine-glutamic acid amino acids inverted, i.e., substrates 3 and 6, were phosphorylated to less than 5% of the level of the wild-type substrate (substrate 1). Substrate 4, which contains the mutant POU DNA-binding domain, was phosphorylated to 50% of the level of the wild-type substrate, i.e., substrate 1. A large difference in the phosphorylation of the substrates was not expected since calf thymus DNA had been used for DNA-PK activation (not the specific Oct-1 binding fragment of the substrate) and the substrate concentrations were relatively high. These control results show that the wild-type recombinant substrate is phosphorylated predominantly at one site by DNA-PK and this site corresponds to the SQ (Ser Gln) site corresponding to the human p53 serine 15 site.

Isoelectrofocusing Analysis of Recombinant DNA-PK Substrates

Samples of the purified wild-type and control recombinant DNA-PK substrates, approximately 1 μg each, were analyzed on a slab isoelectric focusing gel prepared with Bio-Lyte 5/7 ampholytes (Bio-Rad) and urea as described by Copeland (1994). The gel was focused at 100 volts for approximately 30 minutes, then at 200 volts for approximately 4 hours. The scanned image of the Coomassie blue stained gel is shown in Figure 10.

The samples and markers are shown in Figure 10 in the following manner: a)

bovine erythrocyte carbonic anhydrase II, pI 5.4 isoform, b) bovine erythrocyte

carbonic anhydrase II, pI 5.9 isoform, c) human erythrocyte carbonic anhydrase I, pI

6.6 isoform; d) wild-type recombinant substrate (ESQE-DBP+) (substrate 1); e)

control substrate with wild-type DNA-PK site and mutant POU DNA-binding domain

(ESQE-DBP-) (substrate 4); f) control substrate with alanine substituted for serine at the

DNA-PK site (EAQE-DBP+) (substrate 2); g) control substrate with the glutamine-

glutamic acid pair at the DNA-PK site inverted (ESEQ-DBP+) (substrate 3); h) control

substrate with alanine mutation and a mutant POU domain (EAQE-DBP-) (substrate 5);

i) control substrate with QE inversion and a mutant POU domain (ESEQ-DBP-)

(substrate 6); j) glucose oxidase from *A. nigers*; and k) bovine lactoglobulin. The pI

values listed at the right side of Figure 10 are for the markers as the native proteins.

The markers pI values are those specified by the supplier.

As shown in Figure 10, the recombinant protein substrates focused primarily as

single electrophoretic species with pIs (isoelectric points) near 5, as expected from the

sequences of the substrate. The wild-type and mutant POU domain substrates focused

at distinct positions that are baseline resolved. It is believed that this difference is a

consequence of a charge change resulting from the substitution of alanine for arginine

in the mutant POU domain. One skilled in the art would predict a singly

phosphorylated wild-type substrate would focus at a lower position than the substrates

with the mutant POU domain because phosphate provides two negative charges.

A parallel experiment was also conducted (data not shown) in which the

substrates were electrophoretically transferred from the isoelectric focusing gel to a

PVDF membrane. The substrates were then detected with an antibody specific for the

C-terminal HSV epitope. Based upon this experiment, the phosphorylated and

unphosphorylated substrates could be readily distinguished.

Example 17

Construction of mammalian cell expression vectors containing substrate cassettes

The substrate cassettes produced in Example 15 were also moved to a vector

suitable for expression in mammalian cells. The vector pMAMneo, (Clontech Cat

#6104-1, 400 Fabian Way, Palo Alto, CA 94303-4607), was chosen for expressing the

substrate cassettes in eukaryotic cells. The vector pMAMneo is a well characterized

vector for expressing genes in eukaryotic cells. The vector contains a gene for

neomycin resistance. Thus, retention of plasmid sequences can be selected with the

antibiotic G-418. The gene to be expressed is placed under control of the mouse

mammary tumor virus promoter, which is expressed at low levels in the absence of an

inducer and expressed at high levels after the addition of dexamethasone inducer. A

map of pMAMneo is shown in Figure 6.

To convert pMAMneo into a vector suitable for accepting the artificial substrate

expression casette (the *Xba* I - *Not* I fragments from the plasmids of Example 15), the

pMAMneo multiple cloning segment (MCS), which contains sites for *Nhe* I

(G|CTAGC), *Sma* I (CCC|GGG), *Sal* I (G|TCGAC), and *Xho* I (C|TCGAG), was

20   altered to include a *Xba* I site, a *Not* I site, and other sites which are shown in Table

11.

The pMAMneo DNA was cleaved with *Nhe* I and *Xho* I and the intervening

fragment was replaced with a double stranded oligonucleotide prepared from

oligonucleotides #2736 (sense-strand) and #2736 (antisense-strand), which are shown in

Table 11.

<div align="center">Table 11</div>

#2736 pMAMneo MCS expansion oligonucleotide, sense-strand.

5'-CTAGCTCTAGAGGCGCGCCCGGGTACCGCGGCCGCC-3' (SEQ ID NO: 57)

#2737 pMAMneo MCS expansion oligonucleotide, antisense-strand).

5'-TCGAGGCGGCCGCGGTACCCGGGCGCGCCTCTAGAG-3' (SEQ ID NO: 58)

Annealed oligonucleotide and restriction sites:

5'-CTAGCTCTAGAGGCGCGCCCGGGTACCGCGGCCGCC--3'(SEQ ID NO: 57)
   3'-GAGATCTCCGCGCGGGCCCATGGCGCCGGCGGAGCT-5' (SEQ ID NO: 58)
      NheI XbaI AscI SmaI KpnI SacII NotI XhoI

The resulting vector was designated p354 (pMACneo) and the MCS region

contains sites for the following restriction enzymes: *Nde* I (G|CTAGC), *Xba* I

(T|CTAGA), *Asc* I (GG|CGCGCC), *Sma* I (CCC|GGG), *Kpn* I (CCATG|G), *Sac* I

(CCGC|GG), *Not* I (GC|GGCCGC), and *Xho* I (C|TCGAG) (Figure 4: pMACneo

plasmid map). A map of p354 (pMACneo) is shown in Figure 6A.

pMACneo DNA was cleaved with *Xba* I and *Not* I. The resulting vector

fragment was ligated with the *Xba* I - *Not* I fragments from each of the substrate

vectors. The resulting mammalian substrate expression vectors were designated:

p422SUB1 (derivative of p349SUB1), p423SUB2 (derivative of p350SUB2),

p424SUB3 (derivative of p355SUB3), p425SUB4 (derivative of p356SUB4),

p426SUB5 (derivative of p357SUB5), p427SUB6 (derivative of p358SUB6). A

plasmid map of p422SUB1, expressing the wild-type substrate, is shown in Figure 11.

Introduction of the expression vectors into eukaryotic cells, followed by induction of expression of the protein substrates and negative control proteins facilitates the study of DNA-PK activity in the living cells.

While described here are what presently is believed to be the preferred embodiments of the invention, those skilled in the art will realize that changes and modifications may be made to the present invention without departing from the spirit of the invention, and it is intended to claim all such changes and modifications.

CLAIMS:

1. A method for detecting and quantitating DNA-activated protein kinase (DNA-PK) activity in a biological sample, comprising the steps of:

(a) forming a reaction mixture by contacting a biological sample with a detectably-labeled phosphate donor and a synthetic peptide substrate defined by the following features to provide specific recognition and phosphorylation by DNA-PK: (1) a phosphate-accepting amino acid pair which may include serine-glutamine (Ser-Gln) (SQ), threonine-glutamine (Thr-Gln) (TQ), glutamine-serine (Gln-Ser) (QS), or glutamine-threonine (Gln-Thr) (QT); (2) enhancer amino acids which may include glutamic acid or glutamine immediately adjacent at the amino- or carboxyl- side of the amino acid pair and forming an amino acid pair-enhancer unit; (3) a first spacer sequence at the amino terminus of the amino acid pair-enhancer unit; (4) a second spacer sequence at the carboxyl terminus of the amino acid pair-enhancer unit, which spacer sequences may include any combination of amino acids that does not provide a phosphorylation site consensus sequence motif; and (5) a tag moiety, which may be an amino acid sequence or another chemical entity that permits separating the synthetic peptide from the phosphate donor in the reaction mixture;

(b)   incubating said reaction mixture for a time and at a temperature to allow the transfer of phosphate from said phosphate donor to said synthetic peptide substrate;

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(c)   stopping the transfer of phosphate from said phosphate donor to said synthetic peptide substrate;

(d)   determining the amount of phosphate transferred from said phosphate donor to said synthetic peptide substrate; and,

(e)   correlating the amount of phosphate transferred to said synthetic peptide substrate to a concentration of DNA-PK activity in said biological sample.

2.   The method of Claim 1, further comprising contacting said biological sample with a preparation of double-stranded DNA.

3.   The method of Claim 2, wherein said preparation of double-stranded DNA is substantially linear.

4.   The method of Claim 2, wherein said preparation of DNA is provided at a concentration of DNA ranging from about 1 µg/ml to about 1000 µg/ml of said reaction mixture.

5.   The method of Claim 2, wherein said preparation of DNA is provided at a concentration of DNA ranging from about 5 µg/ml to about 15 µg/ml of said reaction mixture.

6.   The method of Claim 2, wherein said preparation of DNA is provided at a concentration of DNA of about 10 µg/ml of said reaction mixture.

7.   The method of Claim 1, wherein said detectably-labeled phosphate donor is selected from the group consisting of gamma labeled $[^{32}P]$-ATP, $[^{32}P]$-dATP, $[^{33}P]$-ATP or $[^{33}P]$-dATP and mixtures thereof.

8.   The method of Claim 7, wherein said detectably-labeled phosphate donor is [^{32}P]-ATP.

9.   The method of Claim 8, wherein said [^{32}P]-ATP is provided at a concentration of ATP ranging from about 20 μM to about 1 mM.

10.   The method of Claim 1, wherein said synthetic peptide substrate is provided at a concentration of substrate ranging from about 50 μM to about 1 mM.

11.   The method of Claim 10, wherein said synthetic peptide substrate is provided at a concentration of substrate ranging from about 100 μM to about 400 μM.

12.   The method of Claim 11, wherein said synthetic peptide substrate is provided at a concentration of substrate of about 200 μM.

13.   The method of Claim 1, wherein said synthetic peptide substrate is selected from the group consisting of Met Glu Glu Pro Gln Ser Asp Pro Ser Val Glu Pro Pro Leu Ser Gln Glu Thr Phe Ser Asp Leu Trp Lys Leu Leu Pro Glu (SEQ ID NO: 1), Met Glu Glu Ser Gln Ser Asp Ile Ser Leu Glu Leu Pro Leu Ser Gln Glu Thr Phe Ser Gly Leu Trp Lys Leu Leu Pro Pro (SEQ ID NO: 2), Met Glu Glu Ser Gln Ser Asp Ile Ser Leu Glu Leu Pro Tyr Lys Lys (SEQ ID NO: 3), Met Glu Glu Pro Gln Ser Asp Pro Ser Val Glu Pro Pro Leu Ser Gln Glu Thr Phe Ser Asp Leu Trp Lys Lys (SEQ ID NO: 4), Asn Asn Val Leu Ser Pro Leu Pro Ser Gln Ala Met Asp Asp Leu Met Lys Lys (SEQ ID NO: 6), Met Ala Ile Tyr Lys Gln Ser Gln His Met Thr Glu Val Val Arg Arg (SEQ ID NO: 7), Glu Pro Pro Leu Ser Gln Glu Thr Phe Ser Asp Leu Trp Lys Lys (SEQ ID NO: 8), Glu Pro Pro Leu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 11), Glu Pro Pro Leu Ser Gln Glu Ala Phe Ala Asp Leu Leu Lys Lys (SEQ ID NO: 12), Glu Pro Pro Gln Ser Leu Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 14), Glu Pro Pro Gln

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Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 15), Glu Pro Pro Leu Thr

Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 16), Glu Pro Pro Asp Ser Gln

Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 17), Pro Glu Glu Ser Gln Glu Ala

Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 18) and Pro Glu Ser Gln Glu Ala Phe Ala

Asp Leu Trp Lys Lys (SEQ ID NO: 19).

14.   The method of Claim 1, wherein said synthetic peptide substrate is

identical to or a variant of the amino acid sequences found at the amino terminus of

human or murine p53 tumor suppressor proteins and which contains Ser[15] of human p53

tumor suppressor protein or Ser[7] or Ser[18] of murine p53 tumor suppressor protein.

15.   The method of Claim 14, wherein said synthetic peptide substrate is

selected from the group consisting of Met Glu Glu Pro Gln Ser Asp Pro Ser Val Glu Pro

Pro Leu Ser Gln Glu Thr Phe Ser Asp Leu Trp Lys Leu Leu Pro Glu (SEQ ID NO: 1),

Met Glu Glu Ser Gln Ser Asp Ile Ser Leu Glu Leu Pro Leu Ser Gln Glu Thr Phe Ser Gly

Leu Trp Lys Leu Leu Pro Pro (SEQ ID NO: 2), Met Glu Glu Ser Gln Ser Asp Ile Ser Leu

Glu Leu Pro Tyr Lys Lys (SEQ ID NO: 3), Met Glu Glu Pro Gln Ser Asp Pro Ser Val

Glu Pro Pro Leu Ser Gln Glu Thr Phe Ser Asp Leu Trp Lys Lys (SEQ ID NO: 4), Glu

Pro Pro Leu Ser Gln Glu Thr Phe Ser Asp Leu Trp Lys Lys (SEQ ID NO: 8), Glu Pro Pro

Leu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 11), Glu Pro Pro Leu

Ser Gln Glu Ala Phe Ala Asp Leu Leu Lys Lys (SEQ ID NO: 12), Glu Pro Pro Gln Ser

Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 15), Glu Pro Pro Asp Ser Gln

Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 17), Pro Glu Glu Ser Gln Glu Ala

Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 18), Pro Glu Ser Gln Glu Ala Phe Ala Asp

Leu Trp Lys Lys (SEQ ID NO: 19), Pro Leu Ser Gln Glu Thr Phe Ser Gly Leu Trp Lys

Leu Leu Pro Pro-Lys Lys (SEQ ID NO: 63), and Pro Leu Ser Gln Glu Ala Phe Ala Gly

Leu Trp Lys Leu Leu Pro Pro-Lys Lys (SEQ ID NO: 64).

16.   The method of Claim 15, wherein said synthetic peptide substrate is selected from the group consisting of Glu Pro Pro Leu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 11), Glu Pro Pro Gln Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 15), Pro Glu Glu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 18) and Pro Glu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 19).

17.   The method of Claim 1, wherein said incubating time ranges from about 1 second to about 60 minutes.

18.   The method of Claim 17, wherein said incubating time ranges from about 2 minutes to about 15 minutes.

19.   The method of Claim 18, wherein said incubating time ranges from about 7 minutes to about 10 minutes.

20.   The method of Claim 1, wherein said incubating is at a temperature ranging from about 5°C to about 45°C.

21.   The method of Claim 20, wherein said incubating is at a temperature ranging from about 20°C to about 37°C.

22.   The method of Claim 21, wherein said incubating is at a temperature of about 30°C.

23.   A composition useful for detecting and quantitating DNA-activated protein kinase (DNA-PK) activity in a biological sample, comprising a synthetic peptide substrate defined by the following features to provide specific recognition and

phosphorylation by DNA-PK: (1) a phosphate-accepting amino acid pair which may include serine-glutamine (Ser-Gln) (SQ), threonine-glutamine (Thr-Gln) (TQ), glutamine-serine (Gln-Ser) (QS), or glutamine-threonine (Gln-Thr) (QT); (2) enhancer amino acids which may include glutamic acid or glutamine immediately adjacent at the amino- or carboxyl- side of the amino acid pair and forming an amino acid pair-enhancer unit; (3) a first spacer sequence at the amino terminus of the amino acid pair-enhancer unit; (4) a second spacer sequence at the carboxyl terminus of the amino acid pair-enhancer unit, which spacer sequences may include any combination of amino acids that does not provide a phosphorylation site consensus sequence motif; and (5) a tag moiety, which may be an amino acid sequence or another chemical entity that permits separating the synthetic peptide from the phosphate donor.

24.   The composition of Claim 23, wherein said first spacer sequence comprises 1 to 4 amino acids.

25.   The composition of Claim 24, wherein said first spacer sequence comprises 1 or 2 amino acids.

26.   The composition of Claim 23, wherein said second spacer sequence comprises at least seven and no more than twenty-three amino acids.

27.   The composition of Claim 26, wherein said second spacer sequence comprises at least seven and no more than eleven amino acids.

28.   The composition of Claim 26, wherein said second spacer sequence comprises seven amino acids.

29.   The composition of Claim 23, wherein said first and second spacer sequences exclude serine, threonine and tyrosine.

30.   The composition of Claim 23, wherein said synthetic peptide substrate is selected from the group consisting of Met Glu Glu Pro Gln Ser Asp Pro Ser Val Glu Pro Pro Leu Ser Gln Glu Thr Phe Ser Asp Leu Trp Lys Leu Leu Pro Glu (SEQ ID NO: 1), Met Glu Glu Ser Gln Ser Asp Ile Ser Leu Glu Leu Pro Leu Ser Gln Glu Thr Phe Ser Gly Leu Trp Lys Leu Leu Pro Pro (SEQ ID NO: 2), Met Glu Glu Ser Gln Ser Asp Ile Ser Leu Glu Leu Pro Tyr Lys Lys (SEQ ID NO: 3), Met Glu Glu Pro Gln Ser Asp Pro Ser Val Glu Pro Pro Leu Ser Gln Glu Thr Phe Ser Asp Leu Trp Lys Lys (SEQ ID NO: 4), Asn Asn Val Leu Ser Pro Leu Pro Ser Gln Ala Met Asp Asp Leu Met Lys Lys (SEQ ID NO: 6), Met Ala Ile Tyr Lys Gln Ser Gln His Met Thr Glu Val Val Arg Arg (SEQ ID NO: 7), Glu Pro Pro Leu Ser Gln Glu Thr Phe Ser Asp Leu Trp Lys Lys (SEQ ID NO: 8), Glu Pro Pro Leu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 11), Glu Pro Pro Leu Ser Gln Glu Ala Phe Ala Asp Leu Leu Lys Lys (SEQ ID NO: 12), Glu Pro Pro Gln Ser Leu Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 14), Glu Pro Pro Gln Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 15), Glu Pro Pro Leu Thr Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 16), Glu Pro Pro Asp Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 17), Pro Glu Glu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 18) and Pro Glu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 19).

31.   The composition of Claim 23, wherein said synthetic peptide substrate is identical to or a variant of the amino acid sequences found at the amino terminus of human or murine p53 tumor suppressor proteins and which contains Ser^{15} of human p53 tumor suppressor protein or Ser^{7} or Ser^{18} of murine p53 tumor suppressor protein.

32.	The composition of Claim 31, wherein said synthetic peptide substrate is selected from the group consisting of Met Glu Glu Pro Gln Ser Asp Pro Ser Val Glu Pro Pro Leu Ser Gln Glu Thr Phe Ser Asp Leu Trp Lys Leu Leu Pro Glu (SEQ ID NO: 1), Met Glu Glu Ser Gln Ser Asp Ile Ser Leu Glu Leu Pro Leu Ser Gln Glu Thr Phe Ser Gly Leu Trp Lys Leu Leu Pro Pro (SEQ ID NO: 2), Met Glu Glu Ser Gln Ser Asp Ile Ser Leu Glu Leu Pro Tyr Lys Lys (SEQ ID NO: 3), Met Glu Glu Pro Gln Ser Asp Pro Ser Val Glu Pro Pro Leu Ser Gln Glu Thr Phe Ser Asp Leu Trp Lys Lys (SEQ ID NO: 4), Glu Pro Pro Leu Ser Gln Glu Thr Phe Ser Asp Leu Trp Lys Lys (SEQ ID NO: 8), Glu Pro Pro Leu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 11), Glu Pro Pro Leu Ser Gln Glu Ala Phe Ala Asp Leu Leu Lys Lys (SEQ ID NO: 12), Glu Pro Pro Gln Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 15), Glu Pro Pro Asp Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 17), Pro Glu Glu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 18), Pro Glu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 19), Pro Leu Ser Gln Glu Thr Phe Ser Gly Leu Trp Lys Leu Leu Pro Pro-Lys Lys (SEQ ID NO: 63), and Pro Leu Ser Gln Glu Ala Phe Ala Gly Leu Trp Lys Leu Leu Pro Pro-Lys Lys (SEQ ID NO: 64).

33.	The composition of Claim 32, wherein said synthetic peptide substrate is selected from the group consisting of Glu Pro Pro Leu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 11), Glu Pro Pro Gln Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 15), Pro Glu Glu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 18) and Pro Glu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 19).

34.   A composition useful for detecting and quantitating DNA-activated protein kinase (DNA-PK) activity in a biological sample, comprising a synthetic peptide substrate selected from the group consisting of Met Glu Glu Pro Gln Ser Asp Pro Ser Val Glu Pro Pro Leu Ser Gln Glu Thr Phe Ser Asp Leu Trp Lys Leu Leu Pro Glu (SEQ ID NO: 1), Met Glu Glu Ser Gln Ser Asp Ile Ser Leu Glu Leu Pro Leu Ser Gln Glu Thr Phe Ser Gly Leu Trp Lys Leu Leu Pro Pro (SEQ ID NO: 2), Met Glu Glu Ser Gln Ser Asp Ile Ser Leu Glu Leu Pro Tyr Lys Lys (SEQ ID NO: 3), Met Glu Glu Pro Gln Ser Asp Pro Ser Val Glu Pro Pro Leu Ser Gln Glu Thr Phe Ser Asp Leu Trp Lys Lys (SEQ ID NO: 4), Glu Pro Pro Leu Ser Gln Glu Thr Phe Ser Asp Leu Trp Lys Lys (SEQ ID NO: 8), Glu Pro Pro Leu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 11), Glu Pro Pro Leu Ser Gln Glu Ala Phe Ala Asp Leu Leu Lys Lys (SEQ ID NO: 12), Glu Pro Pro Gln Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 15), Glu Pro Pro Asp Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 17), Pro Glu Glu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 18) and Pro Glu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 19).

35.   A composition according to Claim 34, wherein the peptide substrate is selected from the group consisting of Glu Pro Pro Leu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 11), Glu Pro Pro Gln Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 15), Pro Glu Glu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 18) and Pro Glu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 19).

36.   A kit for detecting and quantitating DNA-activated protein kinase (DNA-PK) activity, comprising:

(a)   a detectably-labeled phosphate donor;

(b)   a composition useful for specific detection and quantitation of DNA-PK which comprises a synthetic peptide substrate defined by the following features to provide specific recognition and phosphorylation by DNA-PK: (1) a phosphate-accepting amino acid pair which may include serine-glutamine (Ser-Gln) (SQ), threonine-glutamine (Thr-Gln) (TQ), glutamine-serine (Gln-Ser) (QS), or glutamine-threonine (Gln-Thr) (QT); (2) enhancer amino acids which may include glutamic acid or glutamine immediately adjacent at the amino- or carboxyl- side of the amino acid pair and forming an amino acid pair-enhancer unit; (3) a first spacer sequence at the amino terminus of the amino acid pair-enhancer unit; (4) a second spacer sequence at the carboxyl terminus of the amino acid pair-enhancer unit, which spacer sequences may include any combination of amino acids that does not provide a phosphorylation site consensus sequence motif; and (5) a tag moiety, which may be an amino acid sequence or another chemical entity that permits separating the synthetic peptide from the phosphate donor;

(c)   means for detecting a labeled synthetic peptide substrate, whereby detection of labeled synthetic peptide substrate is utilized to determine an amount of DNA-PK activity in said biological sample.

37.   The kit of Claim 36, further including double-stranded DNA.

38.   The kit of Claim 37, wherein said double-stranded DNA is substantitally linear.

39.   The kit of Claim 36, wherein said detectably labeled phosphate donor is selected from the group consisting of gamma labeled [^{32}P]-ATP, [^{32}P]-dATP, [^{33}P]-ATP, [^{33}P]-dATP and mixtures thereof.

40. The kit of Claim 39, wherein said detectably labeled phosphate donor is [^{32}P]-ATP.

41. The kit of Claim 36, wherein said synthetic peptide substrate is selected from the group consisting of Met Glu Glu Pro Gln Ser Asp Pro Ser Val Glu Pro Pro Leu Ser Gln Glu Thr Phe Ser Asp Leu Trp Lys Leu Leu Pro Glu (SEQ ID NO: 1), Met Glu Glu Ser Gln Ser Asp Ile Ser Leu Glu Leu Pro Leu Ser Gln Glu Thr Phe Ser Gly Leu Trp Lys Leu Leu Pro Pro (SEQ ID NO: 2), Met Glu Glu Ser Gln Ser Asp Ile Ser Leu Glu Leu Pro Tyr Lys Lys (SEQ ID NO: 3), Met Glu Glu Pro Gln Ser Asp Pro Ser Val Glu Pro Pro Leu Ser Gln Glu Thr Phe Ser Asp Leu Trp Lys Lys (SEQ ID NO: 4), Asn Asn Val Leu Ser Pro Leu Pro Ser Gln Ala Met Asp Asp Leu Met Lys Lys (SEQ ID NO: 6), Met Ala Ile Tyr Lys Gln Ser Gln His Met Thr Glu Val Val Arg Arg (SEQ ID NO: 7), Glu Pro Pro Leu Ser Gln Glu Thr Phe Ser Asp Leu Trp Lys Lys (SEQ ID NO: 8), Glu Pro Pro Leu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 11), Glu Pro Pro Leu Ser Gln Glu Ala Phe Ala Asp Leu Leu Lys Lys (SEQ ID NO: 12), Glu Pro Pro Gln Ser Leu Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 14), Glu Pro Pro Gln Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 15), Glu Pro Pro Leu Thr Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 16), Glu Pro Pro Asp Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 17), Pro Glu Glu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 18) and Pro Glu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 19).

42. The kit of Claim 36, wherein said synthetic peptide substrate is identical to or a variant of the amino acid sequences found at the amino terminus of human or murine

p53 tumor suppressor proteins and which contains Ser[15] of human p53 tumor suppressor protein or Ser[7] or Ser[18] of murine p53 tumor suppressor protein.

43.   The kit of Claim 42, wherein said synthetic peptide substrate is selected from the group consisting of Met Glu Glu Pro Gln Ser Asp Pro Ser Val Glu Pro Pro Leu Ser Gln Glu Thr Phe Ser Asp Leu Trp Lys Leu Leu Pro Glu (SEQ ID NO: 1), Met Glu Glu Ser Gln Ser Asp Ile Ser Leu Glu Leu Pro Leu Ser Gln Glu Thr Phe Ser Gly Leu Trp Lys Leu Leu Pro Pro (SEQ ID NO: 2), Met Glu Glu Ser Gln Ser Asp Ile Ser Leu Glu Leu Pro Tyr Lys Lys (SEQ ID NO: 3), Met Glu Glu Pro Gln Ser Asp Pro Ser Val Glu Pro Pro Leu Ser Gln Glu Thr Phe Ser Asp Leu Trp Lys Lys (SEQ ID NO: 4), Glu Pro Pro Leu Ser Gln Glu Thr Phe Ser Asp Leu Trp Lys Lys (SEQ ID NO: 8), Glu Pro Pro Leu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 11), Glu Pro Pro Leu Ser Gln Glu Ala Phe Ala Asp Leu Leu Lys Lys (SEQ ID NO: 12), Glu Pro Pro Gln Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 15), Glu Pro Pro Asp Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 17), Pro Glu Glu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 18), Pro Glu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 19), Pro Leu Ser Gln Glu Thr Phe Ser Gly Leu Trp Lys Leu Leu Pro Pro-Lys Lys (SEQ ID NO: 63), and Pro Leu Ser Gln Glu Ala Phe Ala Gly Leu Trp Lys Leu Leu Pro Pro-Lys Lys (SEQ ID NO: 64).

44.   The kit of Claim 43, wherein said synthetic peptide substrate is selected from the group consisting of Glu Pro Pro Leu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 11), Glu Pro Pro Gln Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 15), Pro Glu Glu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 18) and Pro Glu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 19).

45.   The kit of Claim 36, further including a negative control peptide of similar composition to the synthetic peptide substrate which is not phosphorylated by DNA-PK.

46.   The kit of Claim 45, wherein said negative control peptide is identical in amino acid composition to said synthetic peptide substrate.

47.   The kit of Claim 46, wherein said negative control peptide is selected from the group consisting of Glu Pro Pro Leu Ser Glu Gln Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 20) and Pro Glu Ser Glu Gln Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 23).

48.   The kit of Claim 36, wherein said negative control peptide is identical to said synthetic peptide substrate except that the serine or threonine of said amino acid pairs Ser-Gln or Gln-Ser or Thr-Gln or Gln-Thr is replaced with alanine.

49.   The kit of Claim 48, wherein said negative control peptide is selected from the group consisting of Glu Pro Pro Leu Ala Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 21), Pro Glu Glu Ala Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 24) and Pro Glu Glu Ser Glu Gln Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 25).

50.   The kit of Claim 36, further including buffers.

51.   The kit of Claim 36, further including a preparation of DNA-PK.

52.   The kit of Claim 36, further including a reagent to detect a labeled synthetic peptide substrate.

53.   A method for detecting the presence of linear double-stranded DNA in a biological sample, comprising the steps of:

(a)   forming a reaction mixture by contacting a biological sample with a detectably-labeled phosphate donor, a preparation of DNA-PK and a synthetic peptide substrate defined by the following features to provide specific recognition and phosphorylation by DNA-PK: (1) a phosphate-accepting amino acid pair which may include serine-glutamine (Ser-Gln) (SQ), threonine-glutamine (Thr-Gln) (TQ), glutamine-serine (Gln-Ser) (QS), or glutamine-threonine (Gln-Thr) (QT); (2) enhancer amino acids which may include glutamic acid or glutamine immediately adjacent at the amino- or carboxyl- side of the amino acid pair and forming an amino acid pair-enhancer unit; (3) a first spacer sequence at the amino terminus of the amino acid pair-enhancer unit; (4) a second spacer sequence at the carboxyl terminus of the amino acid pair-enhancer unit, which spacer sequences may include any combination of amino acids that does not provide a phosphorylation site consensus sequence motif; and (5) a tag moiety, which may be an amino acid sequence or another chemical entity that permits separating the synthetic peptide from the phosphate donor;

(b)   incubating said reaction mixture for a time and at a temperature to allow the transfer of phosphate from said phosphate donor to said synthetic peptide;

(c)   stopping the transfer of phosphate from said phosphate donor to said synthetic peptide;

(d)   determining the amount of phosphate transferred from said phosphate donor to said synthetic peptide; and

(e)   correlating the amount of phosphate transferred to said synthetic peptide to a concentration of DNA in said biological sample.

54. The method of Claim 53, wherein said biological sample is contacted with said detectably-labeled phosphate donor selected from the group consisting of gamma labeled [^{32}P]-ATP, [^{32}P]-dATP, [^{33}P]-ATP or [^{33}P]-dATP and mixtures thereof.

55. The method of Claim 54, wherein said detectably-labeled phosphate donor is provided at a concentration ranging from about 20μM to about 1 mM.

56. The method of Claim 53, wherein said incubating time ranges from about 1 second to about 60 minutes.

57. The method of Claim 53, wherein said incubating is at a temperature from about 5°C to about 45°C.

58. The method of Claim 53, wherein said preparation of DNA-PK is added to provide a concentration of DNA-PK ranging from about 0.1 U/ml to about 10 U/ml.

59. The method of Claim 53, wherein said synthetic peptide substrate is added to provide a concentration of substrate ranging from about 50 μM to about 1 mM.

60. The method of Claim 53, wherein said biological sample is contacted with said synthetic peptide substrate selected from the group consisting of Met Glu Glu Pro Gln Ser Asp Pro Ser Val Glu Pro Pro Leu Ser Gln Glu Thr Phe Ser Asp Leu Trp Lys Leu Leu Pro Glu (SEQ ID NO: 1), Met Glu Glu Ser Gln Ser Asp Ile Ser Leu Glu Leu Pro Leu Ser Gln Glu Thr Phe Ser Gly Leu Trp Lys Leu Leu Pro Pro (SEQ ID NO: 2), Met Glu Glu Ser Gln Ser Asp Ile Ser Leu Glu Leu Pro Tyr Lys Lys (SEQ ID NO: 3), Met Glu Glu Pro Gln Ser Asp Pro Ser Val Glu Pro Pro Leu Ser Gln Glu Thr Phe Ser Asp Leu Trp Lys Lys (SEQ ID NO: 4), Asn Asn Val Leu Ser Pro Leu Pro Ser Gln Ala Met Asp Asp Leu Met Lys Lys (SEQ ID NO: 6), Met Ala Ile Try Lys Gln Ser Gln His Met Thr Glu Val Val Arg Arg (SEQ ID NO: 7), Glu Pro Pro Leu Ser Gln Glu Thr Phe Ser Asp Leu Trp Lys

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Lys (SEQ ID NO: 8), Glu Pro Pro Leu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys

(SEQ ID NO: 11), Glu Pro Pro Leu Ser Gln Glu Ala Phe Ala Asp Leu Leu Lys Lys (SEQ

ID NO: 12), Glu Pro Pro Gln Ser Leu Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID

NO: 14), Glu Pro Pro Gln Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO:

15), Glu Pro Pro Leu Thr Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 16),

Glu Pro Pro Asp Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 17), Pro

Glu Glu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 18) and Pro Glu

Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 19).

61.   The method of Claim 53, wherein said biological sample is contacted with

said synthetic peptide substrate which is identical to or a variant of the amino acid

sequences found at the amino terminus of human or murine p53 tumor suppressor

proteins and which contains Ser^{15} of human p53 tumor suppressor protein or Ser^7 or Ser^{18}

of murine p53 tumor suppressor protein.

62.   The method of Claim 61, wherein said biological sample is contacted with

a synthetic peptide substrate selected from the group consisting of Met Glu Glu Pro Gln

Ser Asp Pro Ser Val Glu Pro Pro Leu Ser Gln Glu Thr Phe Ser Asp Leu Trp Lys Leu Leu

Pro Glu (SEQ ID NO: 1), Met Glu Glu Ser Gln Ser Asp Ile Ser Leu Glu Leu Pro Leu Ser

Gln Glu Thr Phe Ser Gly Leu Trp Lys Leu Leu Pro Pro (SEQ ID NO: 2), Met Glu Glu

Ser Gln Ser Asp Ile Ser Leu Glu Leu Pro Tyr Lys Lys (SEQ ID NO: 3), Met Glu Glu Pro

Gln Ser Asp Pro Ser Val Glu Pro Pro Leu Ser Gln Glu Thr Phe Ser Asp Leu Trp Lys Lys

(SEQ ID NO: 4), Glu Pro Pro Leu Ser Gln Glu Thr Phe Ser Asp Leu Trp Lys Lys (SEQ

ID NO: 8), Glu Pro Pro Leu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID

NO: 11), Glu Pro Pro Leu Ser Gln Glu Ala Phe Ala Asp Leu Leu Lys Lys (SEQ ID NO:

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12), Glu Pro Pro Gln Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 15),

Glu Pro Pro Asp Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 17), Pro

Glu Glu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 18), Pro Glu Ser

Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 19), Pro Leu Ser Gln Glu Thr

Phe Ser Gly Leu Trp Lys Leu Leu Pro Pro-Lys Lys (SEQ ID NO: 63), and Pro Leu Ser

Gln Glu Ala Phe Ala Gly Leu Trp Lys Leu Leu Pro Pro-Lys Lys (SEQ ID NO: 64).

63.   The method of Claim 62, wherein said biological sample is contacted with

a synthetic peptide substrate selected from the group consisting of Glu Pro Pro Leu Ser

Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 11), Glu Pro Pro Gln Ser Gln

Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 15), Pro Glu Glu Ser Gln Glu Ala

Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 18) and Pro Glu Ser Gln Glu Ala Phe Ala

Asp Leu Trp Lys Lys (SEQ ID NO: 19).

64.   A method for detecting the presence in a sample of a substance that alters

the activity of DNA-PK, comprising the steps of:

(a)   forming a series of reaction mixtures in which increasing amounts of said

sample are contacted with a detectably-labeled phosphate donor, a preparation of DNA-

PK, a preparation of linear double-stranded DNA and a synthetic peptide substrate

defined by the following features to provide specific recognition and phosphorylation by

DNA-PK: (1) a phosphate-accepting amino acid pair which may include serine-glutamine

(Ser-Gln) (SQ), threonine-glutamine (Thr-Gln) (TQ), glutamine-serine (Gln-Ser) (QS), or

glutamine-threonine (Gln-Thr) (QT); (2) enhancer amino acids which may include

glutamic acid or glutamine immediately adjacent at the amino- or carboxyl- side of the

amino acid pair and forming an amino acid pair-enhancer unit; (3) a first spacer sequence

at the amino terminus of the amino acid pair-enhancer unit; (4) a second spacer sequence at the carboxyl terminus of the amino acid pair-enhancer unit, which spacer sequences may include any combination of amino acids that does not provide a phosphorylation site consensus sequence motif; and (5) a tag moiety, which may be an amino acid sequence or another chemical entity that permits separating the synthetic peptide from the phosphate donor;

(b)   incubating said reaction mixtures for a time and at a temperature to allow the transfer of phosphate from said phosphate donor to said synthetic peptide;

(c)   stopping the transfer of phosphate from said phosphate donor to said synthetic peptide;

(d)   determining the amount of phosphate transferred from said phosphate donor to said synthetic peptide in each reaction mixture of said series; and

(e)   correlating the amount of phosphate transferred to said synthetic peptide in each reaction mixture of said series with the presence of a DNA-PK activity altering substance in said sample.

65.   The method of Claim 64, wherein said biological sample is contacted with said detectably-labeled phosphate donor selected from the group consisting of gamma labeled $[^{32}P]$-ATP, $[^{32}P]$-dATP, $[^{33}P]$-ATP or $[^{33}P]$-dATP and mixtures thereof.

66.   The method of Claim 65, wherein said detectably-labeled phosphate donor is provided at a concentration ranging from about 20μM to about 1 mM.

67.   The method of Claim 64, wherein said incubating time ranges from about 1 second to about 60 minutes.

68.   The method of Claim 64, wherein said incubating is at a temperature from about 5°C to about 45°C.

69.   The method of Claim 64, wherein said preparation of DNA-PK is added to provide a concentration of DNA-PK ranging from about 0.1 U/ml to about 10 U/ml.

70.   The method of Claim 64, wherein said synthetic peptide substrate is added to provide a concentration of substrate ranging from about 50 μM to about 1 mM.

71.   The method of Claim 64, wherein said synthetic peptide substrate is selected from the group consisting of Met Glu Glu Pro Gln Ser Asp Pro Ser Val Glu Pro Pro Leu Ser Gln Glu Thr Phe Ser Asp Leu Trp Lys Leu Leu Pro Glu (SEQ ID NO: 1), Met Glu Glu Ser Gln Ser Asp Ile Ser Leu Glu Leu Pro Leu Ser Gln Glu Thr Phe Ser Gly Leu Trp Lys Leu Leu Pro Pro (SEQ ID NO: 2), Met Glu Glu Ser Gln Ser Asp Ile Ser Leu Glu Leu Pro Tyr Lys Lys (SEQ ID NO: 3), Met Glu Glu Pro Gln Ser Asp Pro Ser Val Glu Pro Pro Leu Ser Gln Glu Thr Phe Ser Asp Leu Trp Lys Lys (SEQ ID NO: 4), Asn Asn Val Leu Ser Pro Leu Pro Ser Gln Ala Met Asp Asp Leu Met Lys Lys (SEQ ID NO: 6), Met Ala Ile Try Lys Gln Ser Gln His Met Thr Glu Val Val Arg Arg (SEQ ID NO: 7), Glu Pro Pro Leu Ser Gln Glu Thr Phe Ser Asp Leu Trp Lys Lys (SEQ ID NO: 8), Glu Pro Pro Leu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 11), Glu Pro Pro Leu Ser Gln Glu Ala Phe Ala Asp Leu Leu Lys Lys (SEQ ID NO: 12), Glu Pro Pro Gln Ser Leu Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 14), Glu Pro Pro Gln Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 15), Glu Pro Pro Leu Thr Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 16), Glu Pro Pro Asp Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 17), Pro Glu Glu Ser Gln Glu Ala

Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 18) and Pro Glu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 19).

72.   The method of Claim 64, wherein said biological sample is contacted with said synthetic peptide substrate which is identical to or a variant of the amino acid sequences found at the amino terminus of human or murine p53 tumor suppressor proteins and which contains Ser^{15} of human p53 tumor suppressor protein or Ser^{7} or Ser^{18} of murine p53 tumor suppressor protein.

73.   The method of Claim 72, wherein said biological sample is contacted with a synthetic peptide substrate selected from the group consisting of Met Glu Glu Pro Gln Ser Asp Pro Ser Val Glu Pro Pro Leu Ser Gln Glu Thr Phe Ser Asp Leu Trp Lys Leu Leu Pro Glu (SEQ ID NO: 1), Met Glu Glu Ser Gln Ser Asp Ile Ser Leu Glu Leu Pro Leu Ser Gln Glu Thr Phe Ser Gly Leu Trp Lys Leu Leu Pro Pro (SEQ ID NO: 2), Met Glu Glu Ser Gln Ser Asp Ile Ser Leu Glu Leu Pro Tyr Lys Lys (SEQ ID NO: 3), Met Glu Glu Pro Gln Ser Asp Pro Ser Val Glu Pro Pro Leu Ser Gln Glu Thr Phe Ser Asp Leu Trp Lys Lys (SEQ ID NO: 4), Glu Pro Pro Leu Ser Gln Glu Thr Phe Ser Asp Leu Trp Lys Lys (SEQ ID NO: 8), Glu Pro Pro Leu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 11), Glu Pro Pro Leu Ser Gln Glu Ala Phe Ala Asp Leu Leu Lys Lys (SEQ ID NO: 12), Glu Pro Pro Gln Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 15), Glu Pro Pro Asp Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 17), Pro Glu Glu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 18), Pro Glu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 19), Pro Leu Ser Gln Glu Thr Phe Ser Gly Leu Trp Lys Leu Leu Pro Pro-Lys Lys (SEQ ID NO: 63), and Pro Leu Ser Gln Glu Ala Phe Ala Gly Leu Trp Lys Leu Leu Pro Pro-Lys Lys (SEQ ID NO: 64).

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74.   The method of Claim 73, wherein said biological sample is contacted with a synthetic peptide substrate selected from the group consisting of Glu Pro Pro Leu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 11), Glu Pro Pro Gln Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 15), Pro Glu Glu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 18) and Pro Glu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 19).

75.   The method of Claim 64, wherein said increasing amounts of sample range from zero percent, by volume, of a reaction mixture to about fifty percent, by volume, of a reaction mixture.

76.   The method of Claim 64, wherein said sample comprises a biological sample.

77.   The method of Claim 64, wherein said sample comprises a chemical solution.

78.   A method for detecting the presence of protein phosphatase in a biological sample, comprising the steps of:

(a)   forming a reaction mixture by contacting a biological sample with a phosphorylated synthetic peptide substrate defined by the following features: (1) a phosphorylated amino acid pair which may include phosphoserine-glutamine (PO_4•Ser-Gln), phosphothreonine-glutamine (PO_4•Thr-Gln), glutamine-phosphoserine (Gln-PO_4•Ser), or glutamine-phosphothreonine (Gln-PO_4•Thr); (2) enhancer amino acids which may include glutamic acid or glutamine immediately adjacent at the amino- or carboxyl- side of the amino acid pair and forming an amino acid pair-enhancer unit; (3) a first spacer sequence at the amino terminus of the amino acid pair-enhancer unit; (4) a

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second spacer sequence at the carboxyl terminus of the amino acid pair-enhancer unit,

which spacer sequences may include any combination of amino acids that does not

provide a phosphorylation site consensus sequence motif; and (5) a tag moiety, which

may be an amino acid sequence or another chemical entity that permits separating the

synthetic peptide from the phosphate donor;

(b) incubating said reaction mixture for a time and at a temperature to allow

the cleavage of phosphate from said synthetic peptide;

(c) stopping the cleavage of phosphate from said synthetic peptide;

(d) determining the amount of phosphate cleaved from said synthetic

peptide; and

(e) correlating the amount of phosphate cleaved from said synthetic peptide

to the presence of protein phosphatase in said biological sample.

79. The method of Claim 78, wherein said biological sample is contacted

with said synthetic peptide substrate selected from the group consisting of Met Glu Glu

Pro Gln Ser Asp Pro Ser Val Glu Pro Pro Leu PO_4-Ser Gln Glu Thr Phe Ser Asp Leu

Trp Lys Leu Leu Pro Glu (SEQ ID NO: 1), Met Glu Glu PO_4-Ser Gln Ser Asp Ile Ser

Leu Glu Leu Pro Leu Ser Gln Glu Thr Phe Ser Gly Leu Trp Lys Leu Leu Pro Pro

(SEQ ID NO: 2), Met Glu Glu PO_4-Ser Gln Ser Asp Ile Ser Leu Glu Leu Pro Tyr Lys

Lys (SEQ ID NO: 3), Met Glu Glu Pro Gln Ser Asp Pro Ser Val Glu Pro Pro Leu PO_4-

Ser Gln Glu Thr Phe Ser Asp Leu Trp Lys Lys (SEQ ID NO: 4), Glu Pro Pro Leu

PO_4-Ser Gln Glu Thr Phe Ser Asp Leu Trp Lys Lys (SEQ ID NO: 8), Glu Pro Pro Leu

PO_4-Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 11), Glu Pro Pro

Leu PO_4-Ser Gln Glu Ala Phe Ala Asp Leu Leu Lys Lys (SEQ ID NO: 12), Glu Pro

Pro Gln PO₄-Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 15), Glu

Pro Pro Asp PO₄-Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 17),

Pro Glu Glu PO₄-Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 18),

Pro Glu PO₄-Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 19), Pro

Leu PO₄-Ser Gln Glu Thr Phe Ser Gly Leu Trp Lys Leu Leu Pro Pro-Lys Lys (SEQ ID

NO: 63), and Pro Leu PO₄-Ser Gln Glu Ala Phe Ala Gly Leu Trp Lys Leu Leu Pro

Pro-Lys Lys (SEQ ID NO: 64).

80.   The method of Claim 79, wherein said biological sample is contacted

with a synthetic peptide substrate selected from the group consisting of Glu Pro Pro Leu

PO₄-Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 11), Glu Pro Pro

Gln PO₄-Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 15), Pro Glu

Glu PO₄-Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 18) and Pro Glu

PO₄-Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys (SEQ ID NO: 19).

81.   The method of Claim 78, wherein said synthetic peptide substrate is

added to provide a concentration of substrate ranging from about 50 μm to about 1 mM

of said reaction mixture.

82.   The method of Claim 78, wherein said incubating time ranges from

about 1 second to about 60 minutes.

83.   The method of Claim 78, wherein said incubating temperature ranges

from about 5°C to about 45°C.

84.   A method for monitoring protein kinase activity in a cell, comprising:

(a)   introducing an expression vector into a cell, said expression vector

containing a gene coding for a protein substrate, said protein substrate having a protein

segment containing a phosphorylation site consensus sequence motif specific for said

protein kinase, an optional nuclear localization signal, an optional DNA-binding domain,

and a detectable epitope;

(b)   expressing said protein substrate to provide a phosphorylatable protein

substrate;

(c)   determining the amount of phosphorylated protein substrate; and

(d)   correlating the amount of phosphorylated protein substrate to a

concentration of said protein kinase activity in said cell.

85.   A method for monitoring DNA-activated protein kinase (DNA-PK)

activity in a cell, comprising:

(a)   introducing an expression vector into a cell, said expression vector

containing a gene coding for a protein substrate, said protein substrate having a protein

segment containing a phosphorylation site consensus sequence motif specific for DNA-

PK, a nuclear localization signal, a DNA-binding domain, and a detectable epitope;

(b)   expressing said protein substrate to provide a phosphorylatable protein

substrate;

(c)   determining the amount of phosphorylated protein substrate; and

(d)   correlating the amount of phosphorylated protein substrate to a

concentration of DNA-PK activity in said cell.

86.   The method of Claim 84, wherein said protein substrate further

comprises:

(a)   an epitope for affinity purification ; and

(b)   a cleavage site, said cleavage site permitting excision of said phosphorylation site from said protein segment.

87.   The method of Claim 85, wherein said protein substrate further comprises:

(a)   an epitope for affinity purification; and

(b)   a cleavage site, said cleavage site permitting excision of said phosphorylation site from said protein segment.

88.   A composition for monitoring protein kinase activity in a cell, comprising an expression vector, said expression vector containing a gene coding for a protein substrate, said protein substrate having a protein segment containing a phosphorylation site consensus sequence motif specific for said protein kinase, an optional nuclear localization signal, an optional DNA-binding domain, and a detectable epitope.

89.   A composition for monitoring DNA-activated protein kinase (DNA-PK) activity in a cell, comprising an expression vector, said expression vector containing a gene coding for a protein substrate, said protein substrate having a protein segment containing a phosphorylation site consensus sequence motif specific for DNA-PK, a nuclear localization signal, a DNA-binding domain, and a detectable epitope.

90.   The composition of Claim 89, wherein said expression vector is substantially identical to p422SUB1.

91.   A kit for monitoring protein kinase activity in a cell, comprising:

(a)   an expression vector, said expression vector containing a gene coding for a protein substrate, said protein substrate having a protein segment containing a

phosphorylation site consensus sequence motif specific for said protein kinase, an

optional nuclear localization signal, an optional DNA-binding domain, and a detectable

epitope;

(b)   means for detecting a phosphorylated protein substrate, whereby detection

of said phosphorylated protein substrate is utilized to determine an amount of said protein

kinase activity in said cell.

92.   A kit for monitoring DNA-activated protein kinase (DNA-PK) activity in

a cell, comprising:

(a)   an expression vector, said expression vector containing a gene coding for a

protein substrate, said protein substrate having a protein segment containing a

phosphorylation site consensus sequence motif specific for DNA-PK, a nuclear

localization signal, a DNA-binding domain, and a detectable epitope;

(b)   means for detecting a phosphorylated protein substrate, whereby detection

of said phosphorylated protein substrate is utilized to determine an amount of DNA-PK

activity in said cell.

93.   A method for identifying agents that are able to alter the activity of a

protein kinase in a cell, comprising:

(a)   introducing an expression vector into a first and a second population of

cells, said expression vector containing a gene coding for a protein substrate, said

protein substrate having a protein segment containing a phosphorylation site consensus

sequence motif specific for said protein kinase, an optional nuclear localization signal,

an optional DNA-binding domain, and a detectable epitope;

(b)   contacting said first population of cells with said agent;

(c)   expressing said protein substrate in each population of cells to provide a

10   phosphorylatable protein substrate;

(d)   determining an amount of phosphorylated protein substrate in each

population of cells;

(e)   comparing the amount of phosphorylated protein determined in step (d)

to establish a difference in the amount of phosphorylated protein; and

(f)   correlating said difference in the amount of phosphorylated protein in the

two populations to the ability of said agent to alter the activity of said protein kinase in

a cell.

94.   A method for identifying agents that are able to alter the activity of

DNA-activated protein kinase (DNA-PK) in a cell, comprising:

(a)   introducing an expression vector into a first and a second populations of

cells, said expression vector containing a gene coding for a protein substrate, said

protein substrate having a protein segment containing a phosphorylation site consensus

sequence motif specific for DNA-PK, a nuclear localization signal, a DNA-binding

domain, and a detectable epitope;

(b)   contacting said first population of cells with said agent;

(c)   expressing said protein substrate in each population of cells to provide a

10   phosphorylatable protein substrate;

(d)   determining an amount of phosphorylated protein substrate in each

population of cells;

(e)   comparing the amount of phosphorylated protein determined in step (d)

to establish a difference in the amount of phosphorylated protein; and

(f)   correlating said difference in the amount of phosphorylated protein in the two populations to the ability of said agent to alter the activity of DNA-PK in a cell.

95.   A method for monitoring a protein kinase activity in a cell, comprising:

(a)   introducing an expression vector into a cell, said expression vector containing a gene coding for a protein substrate, said protein substrate having a protein segment containing a phosphorylation site consensus sequence motif specific for said protein kinase, an optional nuclear localization signal, an optional DNA-binding domain, and a detectable epitope;

(b)   expressing said protein substrate to provide a phosphorylatable protein substrate, said phosphorylatable protein substrate being capable of activating expression of a reporter gene to provide a reporter gene product;

(c)   determining an amount of reporter gene product; and

(d)   correlating the amount of reporter gene product to a concentration of said protein kinase activity in said cell.

96.   A method for monitoring DNA-activated protein kinase (DNA-PK) activity in a cell, comprising:

(a)   introducing an expression vector into a cell, said expression vector containing a gene coding for a protein substrate, said protein substrate having a protein segment containing a phosphorylation site consensus sequence motif specific for DNA-PK, a nuclear localization signal, a DNA-binding domain, and a detectable epitope;

(b)   expressing said protein substrate to provide a phosphorylatable protein substrate, said phosphorylatable protein substrate being capable of activating expression of a reporter gene to provide a reporter gene product;

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(c)   determining an amount of reporter gene product; and

(d)   correlating the amount of reporter gene product to a concentration of

DNA-PK activity in said cell.

97.   A method for detecting and quantitating DNA-activated protein kinase

(DNA-PK) activity in a biological sample, comprising the steps of:

(a)   forming a reaction mixture by contacting a biological sample with a

phosphate donor and a synthetic peptide substrate defined by the following features to

provide specific recognition and phosphorylation by DNA-PK: (1) a phosphate-

accepting amino acid pair which may include serine-glutamine (Ser-Gln) (SQ),

threonine-glutamine (Thr-Gln) (TQ), glutamine-serine (Gln-Ser) (QS), or glutamine-

threonine (Gln-Thr) (QT); (2) enhancer amino acids which may include glutamic acid

or glutamine immediately adjacent at the amino- or carboxyl- side of the amino acid

pair and forming an amino acid pair-enhancer unit; (3) a first spacer sequence at the

amino terminus of the amino acid pair-enhancer unit; (4) a second spacer sequence at

the carboxyl terminus of the amino acid pair-enhancer unit, which spacer sequences

may include any combination of amino acids that does not provide a phosphorylation

site consensus sequence motif; and (5) a tag moiety, which may be an amino acid

sequence or another chemical entity that permits separating the synthetic peptide from

the phosphate donor in the reaction mixture;

(b)   incubating said reaction mixture for a time and at a temperature to allow

the transfer of phosphate from said phosphate donor to said synthetic peptide substrate;

(c)   stopping the transfer of phosphate from said phosphate donor to said

20   synthetic peptide substrate;

(d)   separating phosphorylated peptide substrates from said peptide substrate to which no phosphate was transferred;

(e)   determining an amount of phosphorylated synthetic peptide substrate; and,

(f)   correlating said amount of phosphorylated synthetic peptide substrate to a concentration of DNA-PK activity in said biological sample.

98.   The method of Claim 98 in which the phosphate donor is ATP.

99.   A kit for detecting and quantitating DNA-activated protein kinase (DNA-PK) activity, comprising:

(a)   a phosphate donor;

(b)   a composition useful for specific detection and quantitation of DNA-PK which comprises a synthetic peptide substrate defined by the following features to provide specific recognition and phosphorylation by DNA-PK: (1) a phosphate-accepting amino acid pair which may include serine-glutamine (Ser-Gln) (SQ), threonine-glutamine (Thr-Gln) (TQ), glutamine-serine (Gln-Ser) (QS), or glutamine-threonine (Gln-Thr) (QT); (2) enhancer amino acids which may include glutamic acid or glutamine immediately adjacent at the amino- or carboxyl- side of the amino acid pair and forming an amino acid pair-enhancer unit; (3) a first spacer sequence at the amino terminus of the amino acid pair-enhancer unit; (4) a second spacer sequence at the carboxyl terminus of the amino acid pair-enhancer unit, which spacer sequences may include any combination of amino acids that does not provide a phosphorylation site consensus sequence motif; and (5) a tag moiety, which may be an amino acid sequence or another chemical entity that permits separating the synthetic peptide from the phosphate donor;

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(c)   means for detecting a phosphorylated synthetic peptide substrate, whereby

detection of said phosphorylated synthetic peptide substrate is utilized to determine an

amount of DNA-PK activity in said biological sample.

100.   The kit of Claim 99, wherein said phosphate donor is ATP.

101.   The kit of Claim 99, wherein said synthetic peptide substrate is selected

from the group consisting of Glu Pro Pro Leu Ser Gln Glu Ala Phe Ala Asp Leu Trp

Lys Lys (SEQ ID NO: 11), Glu Pro Pro Gln Ser Gln Glu Ala Phe Ala Asp Leu Trp

Lys Lys (SEQ ID NO: 15), Pro Glu Glu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys

Lys (SEQ ID NO: 18) and Pro Glu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys Lys

(SEQ ID NO: 19).

102.   The kit of Claim 99, further including a negative control peptide of

similar compostion to the synthetic peptide substrate which is not phosphorylated by

DNA-PK.

103.   The kit of Claim 99, further including buffers.

104.   The kit of Claim 99, further including a preparaton of DNA-PK.

105.   The kit of Claim 99, further including a reagent to detect a

phosphorylated peptide substrate.

106.   A method for detecting the presence in a sample of a substance that alters

the activity of DNA-PK, comprising the steps of:

(a)   forming a series of reaction mixtures in which increasing amounts of said

sample are contacted with a phosphate donor, a preparation of DNA-PK, a preparation of

linear double-stranded DNA and a synthetic peptide substrate defined by the following

features to provide specific recognition and phosphorylation by DNA-PK: (1) a

phosphate-accepting amino acid pair which may include serine-glutamine (Ser-Gln) (SQ), threonine-glutamine (Thr-Gln) (TQ), glutamine-serine (Gln-Ser) (QS), or glutamine-threonine (Gln-Thr) (QT); (2) enhancer amino acids which may include glutamic acid or glutamine immediately adjacent at the amino- or carboxyl- side of the amino acid pair and forming an amino acid pair-enhancer unit; (3) a first spacer sequence at the amino terminus of the amino acid pair-enhancer unit; (4) a second spacer sequence at the carboxyl terminus of the amino acid pair-enhancer unit, which spacer sequences may include any combination of amino acids that does not provide a phosphorylation site consensus sequence motif; and (5) a tag moiety, which may be an amino acid sequence or another chemical entity that permits separating the synthetic peptide from the phosphate donor;

(b)   incubating said reaction mixtures for a time and at a temperature to allow the transfer of phosphate from said phosphate donor to said synthetic peptide;

(c)   stopping the transfer of phosphate from said phosphate donor to said synthetic peptide;

(d)   determining an amount of phosphorylated synthetic peptide in each reaction mixture of said series; and

(e)   correlating said amount of phosphorylated synthetic peptide in each reaction mixture of said series with the presence of a DNA-PK activity altering substance in said sample.

107.   The method of Claim 106, wherein said phosphate donor is ATP.

108.   The method of Claim 106, wherein said synthetic peptide substrate is selected from the group consisting of Glu Pro Pro Leu Ser Gln Glu Ala Phe Ala Asp

Leu Trp Lys Lys (SEQ ID NO: 11), Glu Pro Pro Gln Ser Gln Glu Ala Phe Ala Asp

Leu Trp Lys Lys (SEQ ID NO: 15), Pro Glu Glu Ser Gln Glu Ala Phe Ala Asp Leu

Trp Lys Lys (SEQ ID NO: 18) and Pro Glu Ser Gln Glu Ala Phe Ala Asp Leu Trp Lys

Lys (SEQ ID NO: 19).

109.   The method of Claim 106, wherein said increasing amounts of sample

range from zero percent, by volume, of a reaction mixture to about fifty percent, by

10   volume, of a reaction mixture.

110.   The method of Claim 106, wherein said sample comprises a biological

sample.

111.   The method of Claim 106, wherein said sample comprises a chemical

solution.

ABSTRACT

DNA-PK ASSAY

The present invention provides a method for detecting DNA-activated protein kinase (DNA-PK) activity in a biological sample. The method includes contacting a biological sample with a detectably-labeled phosphate donor and a synthetic peptide substrate defined by the following features to provide specific recognition and phosphorylation by DNA-PK: (1) a phosphate-accepting amino acid pair which may include serine-glutamine (Ser-Gln) (SQ), threonine-glutamine (Thr-Gln) (TQ), glutamine-serine (Gln-Ser) (QS), or glutamine-threonine (Gln-Thr) (QT); (2) enhancer amino acids which may include glutamic acid or glutamine immediately adjacent at the amino- or carboxyl- side of the amino acid pair and forming an amino acid pair-enhancer unit; (3) a first spacer sequence at the amino terminus of the amino acid pair-enhancer unit; (4) a second spacer sequence at the carboxyl terminus of the amino acid pair-enhancer unit, which spacer sequences may include any combination of amino acids that does not provide a phosphorylation site consensus sequence motif; and, (5) a tag moiety, which may be an amino acid sequence or another chemical entity that permits separating the synthetic peptide from the phosphate donor. A compostion and a kit for the detection of DNA-PK activity are also provided. Methods for detecting

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DNA, protein phosphatases and substances that alter the activity of DNA-PK are also

20   provided. The present invention also provides a method of monitoring protein kinase

and DNA-PK activity in living cells. A composition and a kit for monitoring protein

kinase activity *in vitro* and a composition and a kit for monitoring DNA-PK activities in

living cells are also provided. A method for identifying agents that alter protein kinase

activity *in vitro* and a method for identifying agents that alter DNA-PK activity in living

cells are also provided.

FIGURE 1

Phosphorylation of Synthetic Peptides by Human DNA-PK
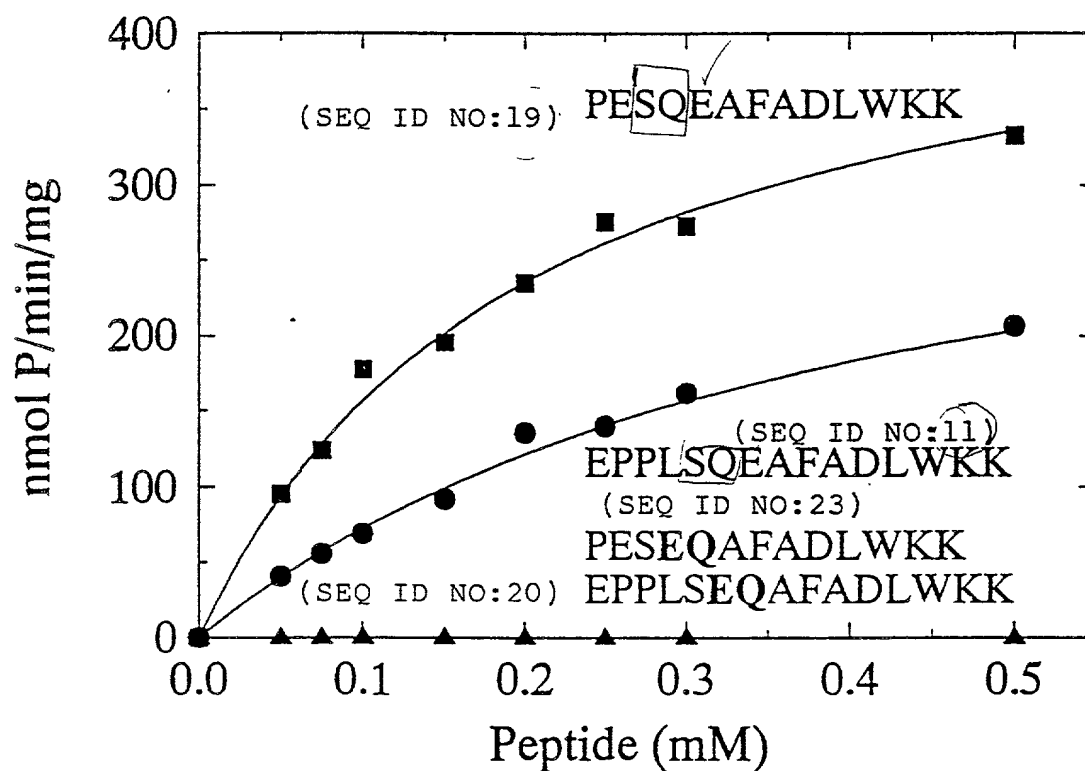
FIGURE 2

Phosphorylation of Synthetic Peptides
by Purified Human DNA-PK

Graph: 1/V (nmol P/min/mg) versus 1/[S] (mM⁻¹)

○ #15=EPPLSQEAFADLWKK
□ #80=PESQEAFADLWKK
(SEQ ID NO:11)
(SEQ ID NO:19)
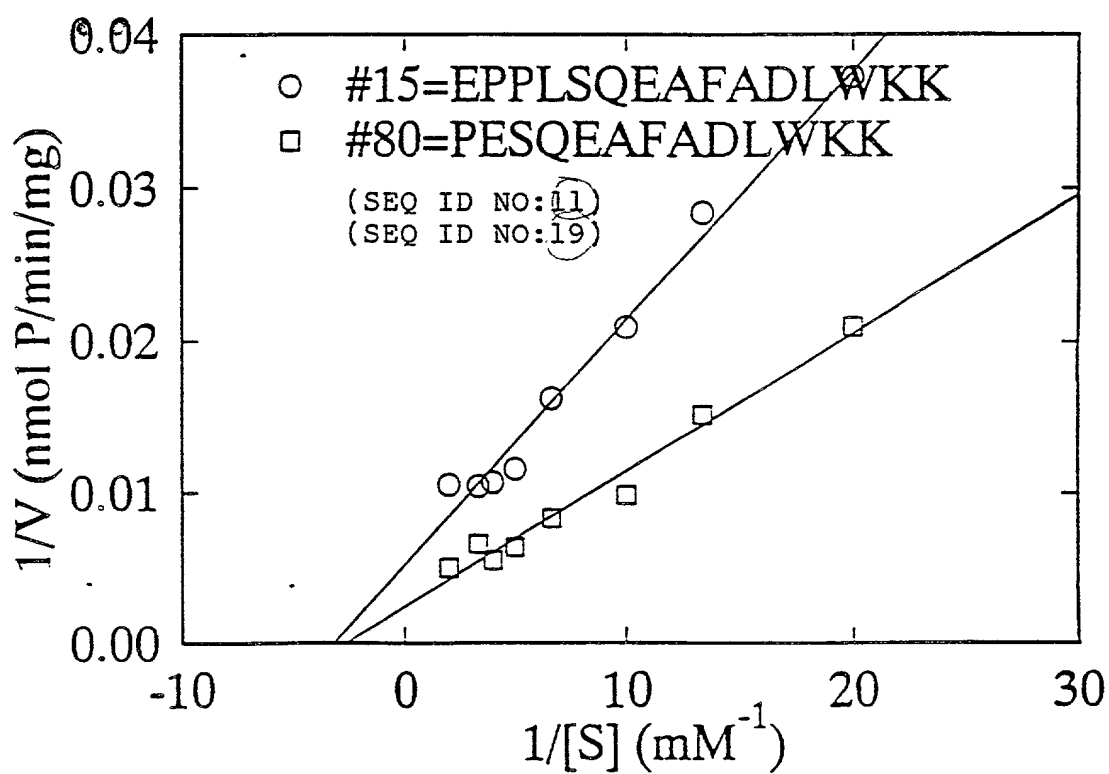
FIGURE 3

Phosphorylation of Synthetic
Peptides by Human DNA-PK

PESQEAFADLWKK (SEQ ID NO:19)
PEESQEAFADLWKK (SEQ ID NO:18)
EPPLSQEAFADLWKK (SEQ ID NO:11)

FIGURE 4

Artificial DNA-PK Substrates

p53 DNA-PK Phosphorylation Motif

6 x His Purification Epitope

Adenovirus EP
Cleavage Site

DNA-Binding
Motif

HSV MAb
Detection
Epitope

ESQE 6 H E P	Oct-1/POU	HSV	Substrate 1
ESQE 6 H E P	R49A	Oct-1/POU	HSV	Substrate 2
EAQE 6 H E P	Oct-1/POU	HSV	Substrate 3
EAQE 6 H E P	R49A	Oct-1/POU	HSV	Substrate 4
ESEQ 6 H E P	Oct-1/POU	HSV	Substrate 5
ESEQ 6 H E P	R49A	Oct-1/POU	HSV	Substrate 6

0   40   80   120   160   201 AA

PT7HPOU1
5005 bps

PvuI, 1003
PstI, 1129

EcoRV, 190
HindIII, 346
EcoRI, 377
AatII, 452

AmpR

5005, BamHI
4974, SalI
4789, EcoRI
4547, XhoI
4544, SacI
4501, NcoI
4484, BstEII
4466, NdeI
4427, XbaI
4369, BglII

HPOU1

4087, SalI

3799, XmaIII
3766, NruI

Tet-frag.

3294, BalI

2674, PvuII

XcaI, 2494

FIGURE 5B

Expressed Protein Product of pT7HPOU1

pT7HPOU1: Expression Vector for Human Oct-1 POU Domain with His6
leader

T7HPOU1   5005 bases, circular

Lab Strain: #236 = pT7HPOU1/DH5[alpha]
Lab Strain: #237 = pT7HPOU1/BL21(DE3)

Plasmid Construction:
    Vector: pT7HIS2 (pET-3 with His6 leader and T7 gene 2.5)
    Cut with Nco I and BamH I
    Insert: POU domain from pET11c-OCT1POU (CWA Strain #234) from
    Winship Herr, Cold Spring Harbor Laboratory. POU
    domain DNA was made by PCR using primers #761 and #430
    (pBR322 EcoR I site). PCR fragment was cut with NcoI
    and BamHI, purified, and inserted in similarly cut
    pT7HIS2 vector (also called pT7AdEP-DBP).

PREDICTED PROTEIN SEQUENCE OF EXPRESSION PRODUCT
Segment: 4469-5005

(SEQ ID NO:59)

Composition

8 Ala	7 Gln	18 Leu	18 Ser
12 Arg	17 Glu	15 Lys	9 Thr
11 Asn	11 Gly	8 Met	2 Trp
6 Asp	6 His	8 Phe	1 Tyr
2 Cys	9 Ile	6 Pro	4 Val

Mol. wt. unmod. chain = 20,352   Number of residues = 178

```
Met Ala Ser Met Thr Gly His His His His His His Gly Met Ser Gly
 1               5                      10                   15

Gly Met Glu Glu Pro Ser Asp Leu Glu Glu Leu Glu Gln Phe Ala Lys
            20              25                      30

Thr Phe Lys Gln Arg Arg Ile Lys Leu Gly Phe Thr Gln Gly Asp Val
        35              40                      45

Gly Leu Ala Met Gly Lys Leu Tyr Gly Asn Asp Phe Ser Gln Thr Thr
    50              55                      60
```

FIGURE 5B (Continued)

(SEQ ID NO:59)

```
Ile Ser Arg Phe Glu Ala Leu Asn Leu Ser Phe Lys Asn Met Cys Lys
65              70              75                      80

Leu Lys Phe Leu Leu Glu Lys Trp Leu Asn Asp Ala Glu Asn Leu Ser
            85              90                      95

Ser Asp Ser Ser Leu Ser Ser Pro Ser Ala Leu Asn Ser Pro Gly Ile
            100             105                 110

Glu Gly Leu Ser Arg Arg Arg Lys Lys Arg Thr Ser Ile Glu Thr Asn
            115             120                 125

Ile Arg Val Leu Glu Lys Ser Phe Leu Glu Asn Gln Lys Pro Thr Ser
    130             135             140

Glu Glu Ile Thr Met Ile Ala Asp Gln Leu Asn Met Glu Lys Glu Val
145             150             155             160

Ile Arg Val Trp Phe Cys Asn Arg Arg Gln Lys Glu Lys Arg Ile Asn
            165             170             175

Pro
```

Reference: Anderson, C. W., and S. P. Lees-Miller. 1992. The nuclear serine/threonine protein kinase DNA-PK. Crit. Rev. Eukaryotic Gene Express. 2, 283-314.

Figure 5C

NUCLEOTIDE SEQUENCE OF pT7HPOU1

(SEQ ID NO:60)

```
GATCCACAGG ACGGGTGTGG TCGCCATGAT CGCGTAGTCG ATAGTGGCTC CAAGTAGCGA   60

AGCGAGCAGG ACTGGGCGGC GGCCAAAGCG GTCGGACAGT GCTCCGAGAA CGGGTGCGCA  120

TAGAAATTGC ATCAACGCAT ATAGCGCTAG CAGCACGCCA TAGTGACTGG CGATGCTGTC  180

GGAATGGACG ATATCCCGCA AGAGGCCCGG CAGTACCGGC ATAACCAAGC CTATGCCTAC  240

AGCATCCAGG GTGACGGTGC CGAGGATGAC GATGAGCGCA TTGTTAGATT TCATACACGG  300

TGCCTGACTG CGTTAGCAAT TTAACTGTGA TAAACTACCG CATTAAAGCT TATCGATGAT  360

AAGCTGTCAA ACATGAGAAT TCTTGAAGAC GAAAGGGCCT CGTGATACGC CTATTTTTAT  420

AGGTTAATGT CATGATAATA ATGGTTTCTT AGACGTCAGG TGGCACTTTT CGGGGAAATG  480

TGCGCGGAAC CCCTATTTGT TTATTTTTCT AAATACATTC AAATATGTAT CCGCTCATGA  540

GACAATAACC CTGATAAATG CTTCAATAAT ATTGAAAAAG GAAGAGTATG AGTATTCAAC  600

ATTTCCGTGT CGCCCTTATT CCCTTTTTTG CGGCATTTTG CCTTCCTGTT TTTGCTCACC  660

CAGAAACGCT GGTGAAAGTA AAAGATGCTG AAGATCAGTT GGGTGCACGA GTGGGTTACA  720

TCGAACTGGA TCTCAACAGC GGTAAGATCC TTGAGAGTTT CGCCCCGAA GAACGTTTTC  780

CAATGATGAG CACTTTTAAA GTTCTGCTAT GTGGCGCGGT ATTATCCCGT GTTGACGCCG  840

GGCAAGAGCA ACTCGGTCGC CGCATACACT ATTCTCAGAA TGACTTGGTT GAGTACTCAC  900

CAGTCACAGA AAAGCATCTT ACGGATGGCA TGACAGTAAG AGAATTATGC AGTGCTGCCA  960

TAACCATGAG TGATAACACT GCGGCCAACT TACTTCTGAC AACGATCGGA GGACCGAAGG 1020

AGCTAACCGC TTTTTTGCAC AACATGGGGG ATCATGTAAC TCGCCTTGAT CGTTGGGAAC 1080

CGGAGCTGAA TGAAGCCATA CCAAACGACG AGCGTGACAC CACGATGCCT GCAGCAATGG 1140

CAACAACGTT GCGCAAACTA TTAACTGGCG AACTACTTAC TCTAGCTTCC CGGCAACAAT 1200

TAATAGACTG GATGGAGGCG GATAAAGTTG CAGGACCACT TCTGCGCTCG GCCCTTCCGG 1260

CTGGCTGGTT TATTGCTGAT AAATCTGGAG CCGGTGAGCG TGGGTCTCGC GGTATCATTG 1320

CAGCACTGGG GCCAGATGGT AAGCCCTCCC GTATCGTAGT TATCTACACG ACGGGGAGTC 1380
```

Figure 5C (Continued)

(SEQ ID NO:60)

```
AGGCAACTAT GGATGAACGA AATAGACAGA TCGCTGAGAT AGGTGCCTCA CTGATTAAGC 1440

ATTGGTAACT GTCAGACCAA GTTTACTCAT ATATACTTTA GATTGATTTA AAACTTCATT 1500

TTTAATTTAA AAGGATCTAG GTGAAGATCC TTTTTGATAA TCTCATGACC AAAATCCCTT 1560

AACGTGAGTT TTCGTTCCAC TGAGCGTCAG ACCCCGTAGA AAAGATCAAA GGATCTTCTT 1620

GAGATCCTTT TTTTCTGCGC GTAATCTGCT GCTTGCAAAC AAAAAAACCA CCGCTACCAG 1680

CGGTGGTTTG TTTGCCGGAT CAAGAGCTAC CAACTCTTTT TCCGAAGGTA ACTGGCTTCA 1740

GCAGAGCGCA GATACCAAAT ACTGTCCTTC TAGTGTAGCC GTAGTTAGGC CACCACTTCA 1800

AGAACTCTGT AGCACCGCCT ACATACCTCG CTCTGCTAAT CCTGTTACCA GTGGCTGCTG 1860

CCAGTGGCGA TAAGTCGTGT CTTACCGGGT TGGACTCAAG ACGATAGTTA CCGGATAAGG 1920

CGCAGCGGTC GGGCTGAACG GGGGGTTCGT GCACACAGCC CAGCTTGGAG CGAACGACCT 1980

ACACCGAACT GAGATACCTA CAGCGTGAGC ATTGAGAAAG CGCCACGCTT CCCGAAGGGA 2040

GAAAGGCGGA CAGGTATCCG GTAAGCGGCA GGGTCGGAAC AGGAGAGCGC ACGAGGGAGC 2100

TTCCAGGGGG AAACGCCTGG TATCTTTATA GTCCTGTCGG GTTTCGCCAC CTCTGACTTG 2160

AGCGTCGATT TTTGTGATGC TCGTCAGGGG GGCGGAGCCT ATGGAAAAAC GCCAGCAACG 2220

CGGCCTTTTT ACGGTTCCTG GCCTTTTGCT GGCCTTTTGC TCACATGTTC TTTCCTGCGT 2280

TATCCCCTGA TTCTGTGGAT AACCGTATTA CCGCCTTTGA GTGAGCTGAT ACCGCTCGCC 2340

GCAGCCGAAC GACCGAGCGC AGCGAGTCAG TGAGCGAGGA AGCGGAAGAG CGCCTGATGC 2400

GGTATTTTCT CCTTACGCAT CTGTGCGGTA TTTCACACCG CATATATGGT GCACTCTCAG 2460

TACAATCTGC TCTGATGCCG CATAGTTAAG CCAGTATACA CTCCGCTATC GCTACGTGAC 2520

TGGGTCATGG CTGCGCCCCG ACACCCGCCA ACACCCGCTG ACGCGCCCTG ACGGGCTTGT 2580

CTGCTCCCGG CATCCGCTTA CAGACAAGCT GTGACCGTCT CCGGGAGCTG CATGTGTCAG 2640

AGGTTTTCAC CGTCATCACC GAAACGCGCG AGGCAGCTGC GGTAAAGCTC ATCAGCGTGG 2700

TCGTGAAGCG ATTCACAGAT GTCTGCCTGT TCATCCGCGT CCAGCTCGTT GAGTTTCTCC 2760
```

(SEQ ID NO:60)

AGAAGCGTTA ATGTCTGGCT TCTGATAAAG CGGGCCATGT TAAGGGCGGT TTTTTCCTGT 2820

TTGGTCACTG ATGCCTCCGT GTAAGGGGGA TTTCTGTTCA TGGGGGTAAT GATACCGATG 2880

AAACGAGAGA GGATGCTCAC GATACGGGTT ACTGATGATG AACATGCCCG GTTACTGGAA 2940

CGTTGTGAGG GTAAACAACT GGCGGTATGG ATGCGGCGGG ACCAGAGAAA AATCACTCAG 3000

GGTCAATGCC AGCGCTTCGT TAATACAGAT GTAGGTGTTC CACAGGGTAG CCAGCAGCAT 3060

CCTGCGATGC AGATCCGGAA CATAATGGTG CAGGGCGCTG ACTTCCGCGT TTCCAGACTT 3120

TACGAAACAC GGAAACCGAA GACCATTCAT GTTGTTGCTC AGGTCGCAGA CGTTTTGCAG 3180

CAGCAGTCGC TTCACGTTCG CTCGCGTATC GGTGATTCAT TCTGCTAACC AGTAAGGCAA 3240

CCCCGCCAGC CTAGCCGGGT CCTCAACGAC AGGAGCACGA TCATGCGCAC CCGTGGCCAG 3300

GACCCAACGC TGCCCGAGAT GCGCCGCGTG CGGCTGCTGG AGATGGCGGA CGCGATGGAT 3360

ATGTTCTGCC AAGGGTTGGT TTGCGCATTC ACAGTTCTCC GCAAGAATTG ATTGGCTCCA 3420

ATTCTTGGAG TGGTGAATCC GTTAGCGAGG TGCCGCCGGC TTCCATTCAG GTCGAGGTGG 3480

CCCGGCTCCA TGCACCGCGA CGCAACGCGG GGAGGCAGAC AAGGTATAGG GCGGCGCCTA 3540

CAATCCATGC CAACCCGTTC CATGTGCTCG CCGAGGCGGC ATAAATCGCC GTGACGATCA 3600

GCGGTCCAGT GATCGAAGTT AGGCTGGTAA GAGCCGCGAG CGATCCTTGA AGCTGTCCCT 3660

GATGGTCGTC ATCTACCTGC CTGGACAGCA TGGCCTGCAA CGCGGGCATC CCGATGCCGC 3720

CGGAAGCGAG AAGAATCATA ATGGGGAAGG CCATCCAGCC TCGCGTCGCG AACGCCAGCA 3780

AGACGTAGCC CAGCGCGTCG GCCGCCATGC CGGCGATAAT GGCCTGCTTC TCGCCGAAAC 3840

GTTTGGTGGC GGGACCAGTG ACGAAGGCTT GAGCGAGGGC GTGCAAGATT CCGAATACCG 3900

CAAGCGACAG GCCGATCATC GTCGCGCTCC AGCGAAAGCG GTCCTCGCCG AAAATGACCC 3960

AGAGCGCTGC CGGCACCTGT CCTACGAGTT GCATGATAAA GAAGACAGTC ATAAGTGCGG 4020

CGACGATAGT CATGCCCCGC GCCCACCGGA AGGAGCTGAC TGGGTTGAAG GCTCTCAAGG 4080

GCATCGGTCG ACGCTCTCCC TTATGCGACT CCTGCATTAG GAAGCAGCCC AGTAGTAGGT 4140

Figure 5C (Continued)

(SEQ ID NO:60)

```
TGAGGCCGTT GAGCACCGCC GCCGCAAGGA ATGGTGCATG CAAGGAGATG GCGCCCAACA 4200

GTCCCCCGGC CACGGGGCCT GCCACCATAC CCACGCCGAA ACAAGCGCTC ATGAGCCCGA 4260

AGTGGCGAGC CCGATCTTCC CCATCGGTGA TGTCGGCGAT ATAGGCGCCA GCAACCGCAC 4320

CTGTGGCGCC GGTGATGCCG GCCACGATGC GTCCGGCGTA GAGGATCGAG ATCTCGATCC 4380

CGCGAAATTA ATACGACTCA CTATAGGGAG ACCACAACGG TTTCCCTCTA GAAATAATTT 4440

TGTTTAACTT TAAGAAGGAG ATATACAT ATG GCT TCT ATG ACT GGT CAC CAC      4492

CAC CAT CAC CAT GGT ATG AGC GGC GGC ATG GAG GAG CCC AGT GAC CTT      4540

GAG GAG CTC GAG CAG TTT GCC AAG ACC TTC AAA CAA AGA CGA ATC AAA      4588

CTT GGA TTC ACT CAG GGT GAT GTT GGG CTC GCT ATG GGG AAA CTA TAT      4636

GGA AAT GAC TTC AGC CAA ACT ACC ATC TCT CGA TTT GAA GCC TTG AAC      4684

CTC AGC TTT AAG AAC ATG TGC AAG TTG AAG CCA CTT TTA GAG AAG TGG      4732

CTA AAT GAT GCA GAG AAC CTC TCA TCT GAT TCG TCC CTC TCC AGC CCA      4780

AGT GCC CTG AAT TCT CCA GGA ATT GAG GGC TTG AGC AGG CGC AGG AAG      4828

AAA CGC ACC AGC ATA GAG ACC AAC ATC CGT GTG GCC TTA GAG AAG AGT      4876

TTC TTG GAG AAT CAA AAG CCT ACC TCG GAA GAG ATC ACT ATG ATT GCT      4924

GAT CAG CTC AAT ATG GAA AAA GAG GTG ATT CGT GTT TGG TTC TGT AAC      4972

CGT CGA CAG AAA GAA AAA AGA ATC AAC CCA TAG                          5005
```

FIGURE 6

FIGURE 7

pMACSUB1
9070 bps

Plasmid map of p349SUB1, 5873 bps, showing restriction sites: SmaI,1259; ClaI,1441; PvuII,2744; 3327,EcoO109; 3743,PvuII; 3836,PvuII; 3986,EcoRV; 4227,ApaI; 5001,EcoO109; 5226,XbaI; 5233,SalI; 5261,Bsu36I; 5287,BglII; 5369,SacI; 5372,XhoI; 5614,EcoRI; 5799,SalI; 5863,BamHI; 5869,EcoRI,SacI,2; SalI,9; HindIII,15; NotI,21; XhoI,30; EcoO109,135. Features: KanR, WT-SUB1, lacI.
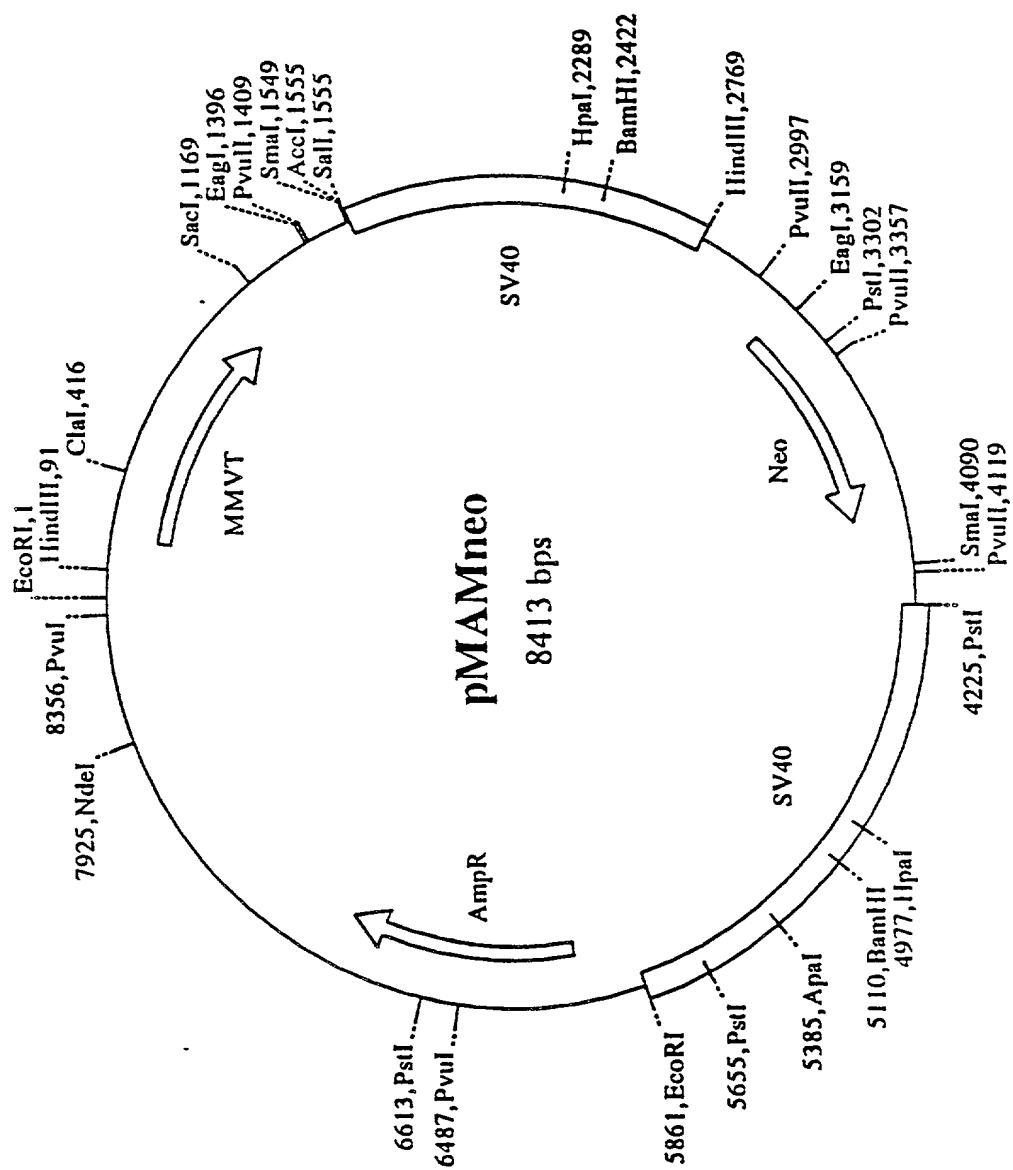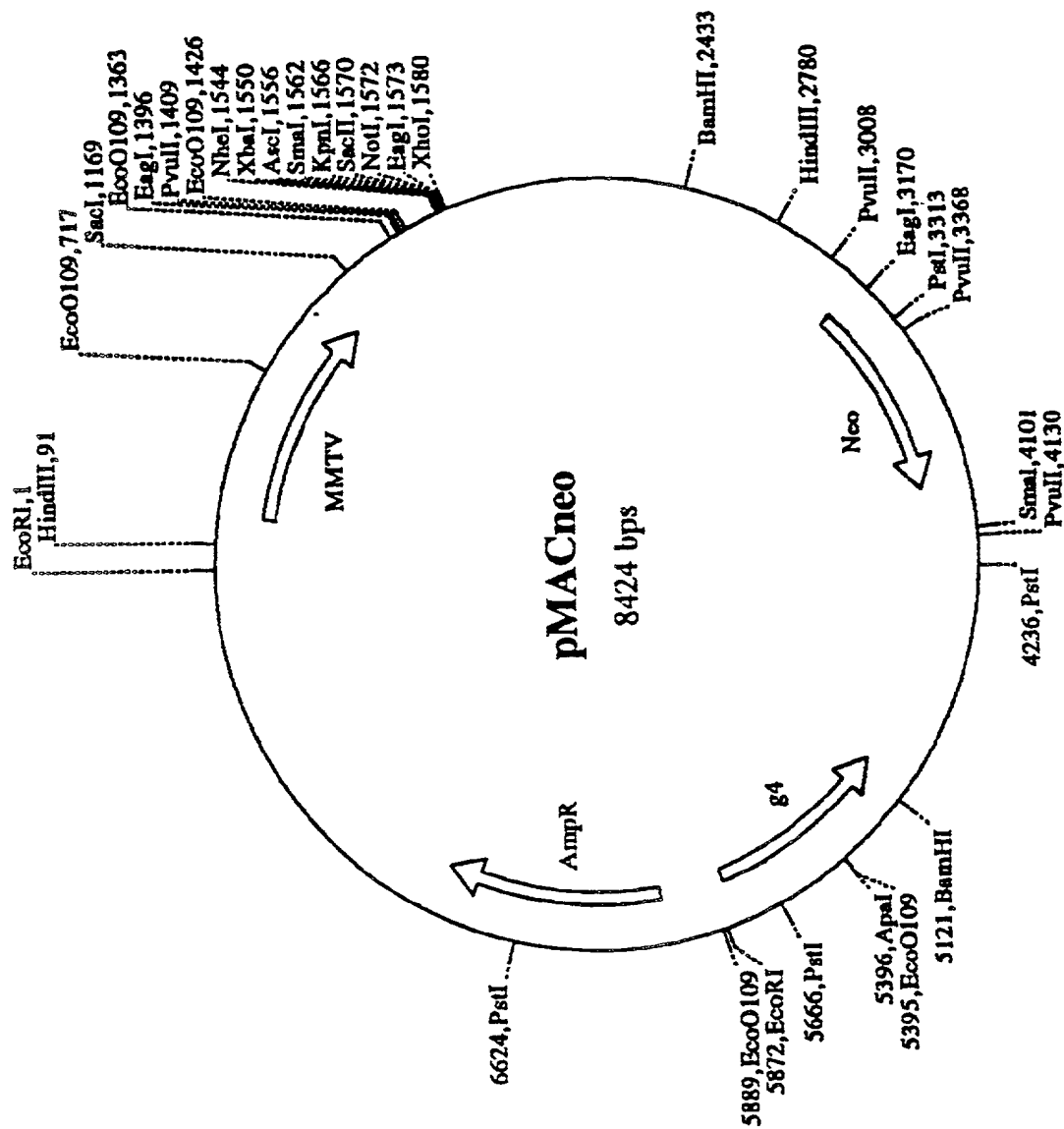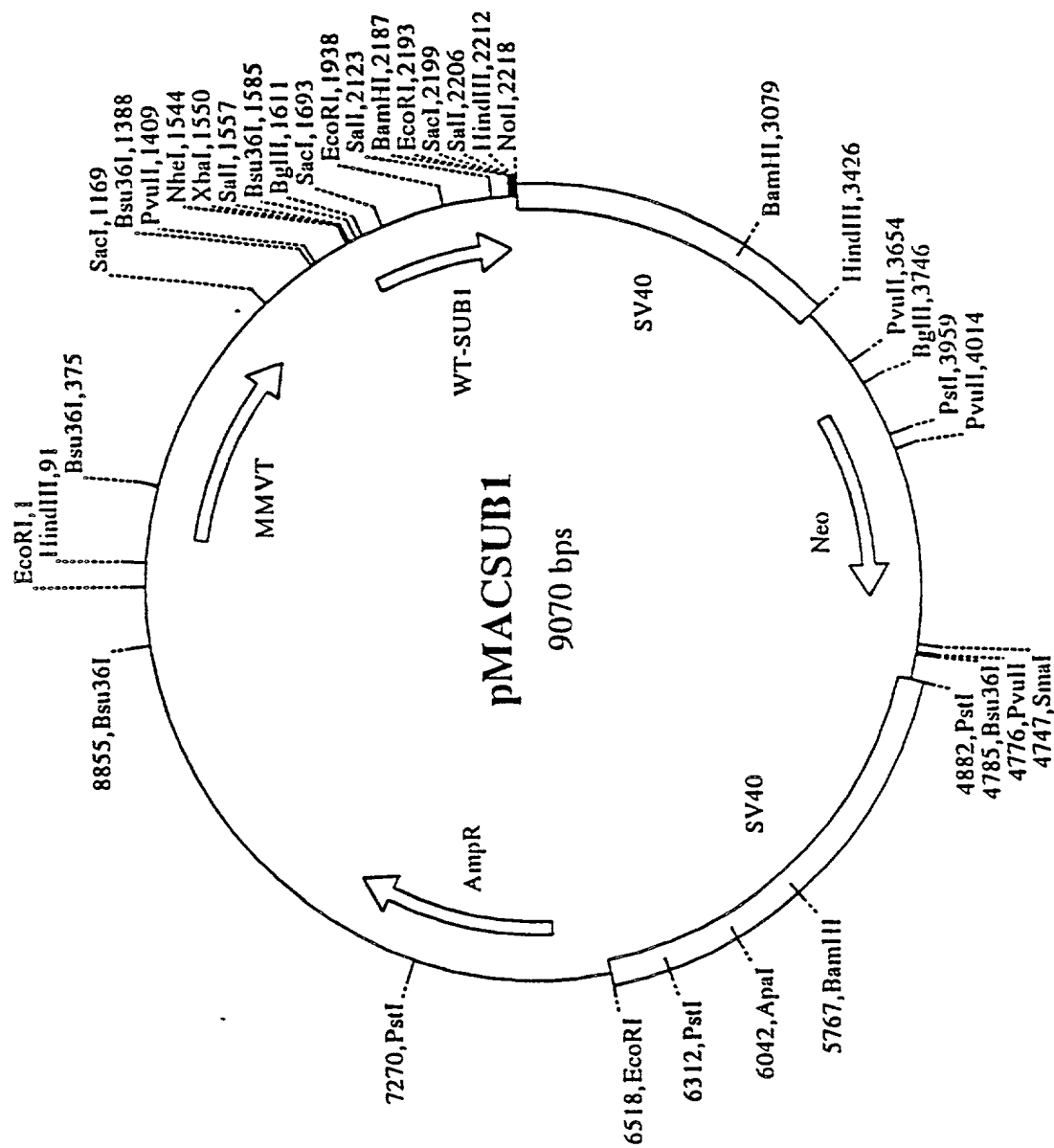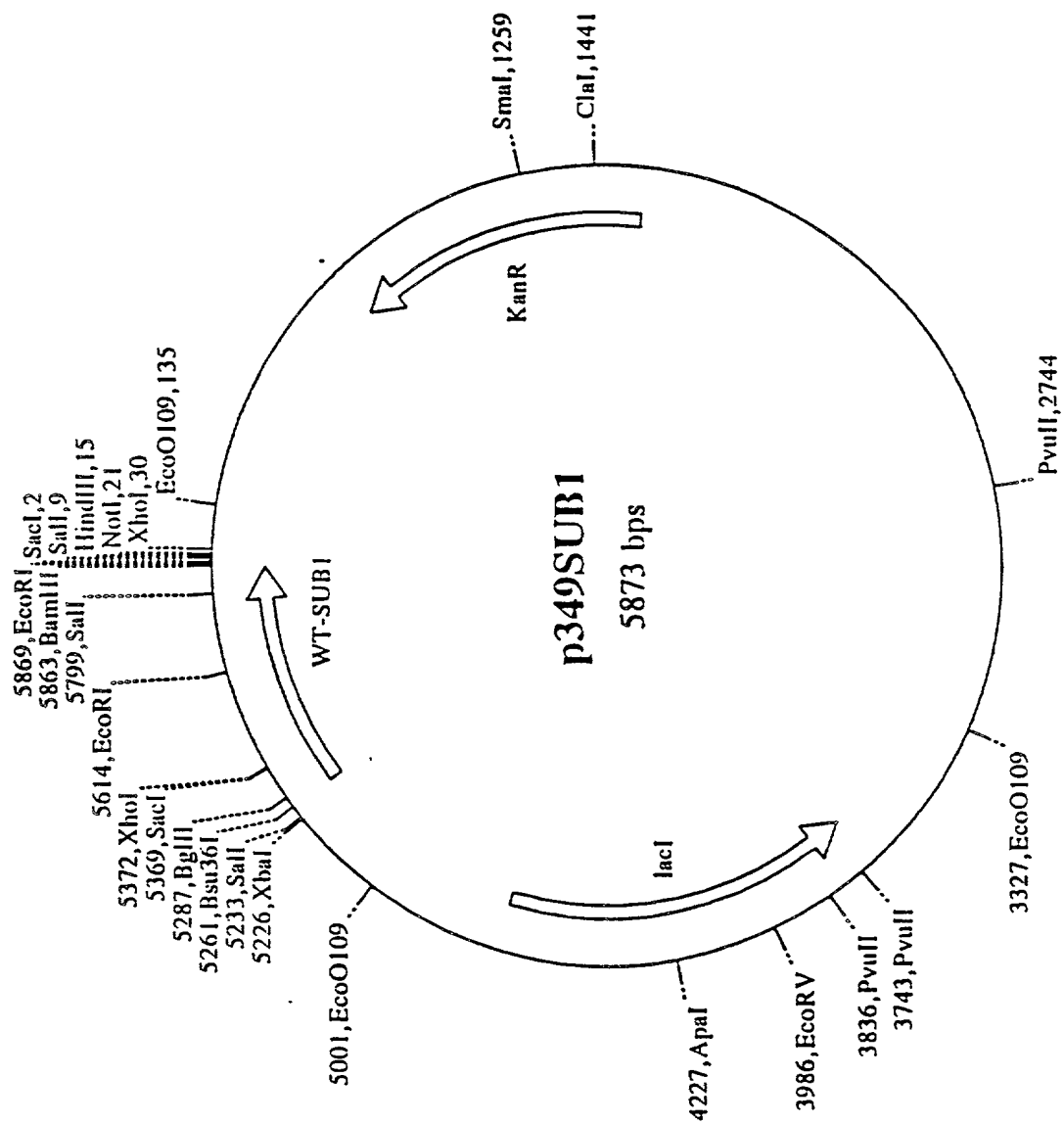
FIGURE 8B

Wild-Type Artificial DNA-PK Substrate 1

Lab Stain: #349 = p349SUB1 in DH5[alpha]
Lab Strain #351 = p349SUB1 in BL21(DE3)

Plasmid Construction:
  VECTOR: p410 = derivative pET-28a (Novagen) without BglII site
  INSERT: Substrate encoding XbaI-BamHI fragment was excised from
       p345 with XbaI and BamHI and cloned into XbaI and BamHI
       cleaved p410.
  ANTIBIOTIC SELECTION: 50 ug/ml Kanamycin

PREDICTED SEQUENCE POUSUB1 ARTIFICIAL DNA-PK SUBSTRATE

Segment: 5258-5860

(SEQ ID NO: 61)

Composition

8 Ala	9 Gln	22 Leu	18 Ser
12 Arg	24 Glu	16 Lys	9 Thr
11 Asn	11 Gly	7 Met	3 Trp
9 Asp	6 His	9 Phe	1 Tyr
2 Cys	9 Ile	11 Pro	4 Val

Mol. wt. unmod. chain = 23,126   Number of residues = 201

```
Met Pro Glu Glu Ser Gln Glu Thr Phe Glu Asp Leu Trp Lys Leu Leu
 1                5                  10                  15

Pro Gly His His His His His His Gly Met Ser Gly Gly Met Glu Glu
             20                  25                  30

Pro Ser Asp Leu Glu Glu Leu Glu Gln Phe Ala Lys Thr Phe Lys Gln
         35              40                  45

Arg Arg Ile Lys Leu Gly Phe Thr Gln Gly Asp Val Gly Leu Ala Met
     50              55                  60

Gly Lys Leu Tyr Gly Asn Asp Phe Ser Gln Thr Thr Ile Ser Arg Phe
 65              70                  75                      80

Glu Ala Leu Asn Leu Ser Phe Lys Asn Met Cys Lys Leu Lys Pro Leu
                 85                  90                  95

Leu Glu Lys Trp Leu Asn Asp Ala Glu Asn Leu Ser Ser Asp Ser Ser
             100                 105                 110
```

(SEQ ID NO:61)

```
Leu Ser Ser Pro Ser Ala Leu Asn Ser Pro Gly Ile Glu Gly Leu Ser
        115         120             125

Arg Arg Arg Lys Lys Arg Thr Ser Ile Glu Thr Asn Ile Arg Val Ala
    130             135             140

Leu Glu Lys Ser Phe Leu Glu Asn Gln Lys Pro Thr Ser Glu Glu Ile
145             150             155             160

Thr Met Ile Ala Asp Gln Leu Asn Met Glu Lys Glu Val Ile Arg Val
            165             170             175

Trp Phe Cys Asn Arg Arg Gln Lys Glu Lys Arg Ile Asn Pro Gln Pro
            180             185             190

Glu Leu Ala Pro Glu Asp Pro Glu Asp
        195             200
```

NOTES:
```
  Ser 5-Gln 6        = Artificial DNA-PK Site
  Glu 10             = Introduced to make BglII site, =Ser in p53,
                       =Ala in peptide
  His 19-His 24      = His tag for Zn/Ni-affinity chromatography
  Met 26-Gly 29      = Adenovirus Proteinase Cleavage Motif
  Glu 31-Pro 190     = POU specific domain of Human Oct-1
  Gln 191-Asp 201    = Novagen HSV Epitope Tag
```

Figure 8C

NUCLEOTIDE SEQUENCE OF p349SUB1

(SEQ ID NO:62)

```
CGAGCTCCGT CGACAAGCTT GCGGCCGCAC TCGAGCACCA CCACCACCAC CACTGAGATC    60

CGGCTGCTAA CAAAGCCCGA AAGGAAGCTG AGTTGGCTGC TGCCACCGCT GAGCAATAAC   120

TAGCATAACC CCTTGGGGCC TCTAAACGGG TCTTGAGGGG TTTTTTGCTG AAAGGAGGAA   180

CTATATCCGG ATTGGCGAAT GGGACGCGCC CTGTAGCGGC GCATTAAGCG CGGCGGGTGT   240

GGTGGTTACG CGCAGCGTGA CCGCTACACT TGCCAGCGCC CTAGCGCCCG CTCCTTTCGC   300

TTTCTTCCCT TCCTTTGTCG CCACGTTCGC CGGCTTTCCC CGTCAAGCTC TAAATCGGGG   360

GCTCCCTTTA GGGTTCCGAT TTAGTGCTTT ACGGCACCTC GACCCCAAAA AACTTGATTA   420

GGGTGATGGT TCACGTAGTG GGCCATCGCC CTGATAGACG GTTTTTCGCC CTTTGACGTT   480

GGAGTCCACG TTCTTTAATA GTGGACTCTT GTTCCAAACT GGAACAACAC TCAACCCTAT   540

CTCGGTCTAT TCTTTTGATT TATAAGGGAT TTTGCCGATT TCGGCCTATT GGTTAAAAAA   600

TGAGCTGATT TAACAAAAAT TTAACGCGAA TTTTAACAAA ATATTAACGT TTACAATTTC   660

AGGTGGCACT TTTCGGGGAA ATGTGCGCGG AACCCCTATT TGTTTATTTT TCTAAATACA   720

TTCAAATATG TATCCGCTCA TGAATTAATT CTTAGAAAAA CTCATCGAGC ATCAAATGAA   780

ACTGCAATTT ATTCATATCA GGATTATCAA TACCATATTT TTGAAAAAGC CGTTTCTGTA   840

ATGAAGGAGA AAACTCACCG AGGCAGTTCC ATAGGATGGC AAGATCCTGG TATCGGTCTG   900

CGATTCCGAC TCGTCCAACA TCAATACAAC CTATTAATTT CCCCTCGTCA AAAATAAGGT   960

TATCAAGTGA GAAATCACCA TGAGTGACGA CTGAATCCGG TGAGAATGGC AAAAGTTTAT  1020

GCATTTCTTT CCAGACTTGT TCAACAGGCC AGCCATTACG CTCGTCATCA AAATCACTCG  1080

CATCAACCAA ACCGTTATTC ATTCGTGATT GCGCCTGAGC GAGACGAAAT ACGCGATCGC  1140

TGTTAAAAGG ACAATTACAA ACAGGAATCG AATGCAACCG GCGCAGGAAC ACTGCCAGCG  1200

CATCAACAAT ATTTTCACCT GAATCAGGAT ATTCTTCTAA TACCTGGAAT GCTGTTTTCC  1260

CGGGGATCGC AGTGGTGAGT AACCATGCAT CATCAGGAGT ACGGATAAAA TGCTTGATGG  1320

TCGGAAGAGG CATAAATTCC GTCAGCCAGT TTAGTCTGAC CATCTCATCT GTAACATCAT  1380
```

(SEQ ID NO:62)

```
TGGCAACGCT ACCTTTGCCA TGTTTCAGAA ACAACTCTGG CGCATCGGGC TTCCCATACA 1440

ATCGATAGAT TGTCGCACCT GATTGCCCGA CATTATCGCG AGCCCATTTA TACCCATATA 1500

AATCAGCATC CATGTTGGAA TTTAATCGCG GCCTAGAGCA AGACGTTTCC CGTTGAATAT 1560

GGCTCATAAC ACCCCTTGTA TTACTGTTTA TGTAAGCAGA CAGTTTTATT GTTCATGACC 1620

AAAATCCCTT AACGTGAGTT TTCGTTCCAC TGAGCGTCAG ACCCCGTAGA AAAGATCAAA 1680

GGATCTTCTT GAGATCCTTT TTTTCTGCGC GTAATCTGCT GCTTGCAAAC AAAAAAACCA 1740

CCGCTACCAG CGGTGGTTTG TTTGCCGGAT CAAGAGCTAC CAACTCTTTT TCCGAAGGTA 1800

ACTGGCTTCA GCAGAGCGCA GATACCAAAT ACTGTCCTTC TAGTGTAGCC GTAGTTAGGC 1860

CACCACTTCA AGAACTCTGT AGCACCGCCT ACATACCTCG CTCTGCTAAT CCTGTTACCA 1920

GTGGCTGCTG CCAGTGGCGA TAAGTCGTGT CTTACCGGGT TGGACTCAAG ACGATAGTTA 1980

CCGGATAAGG CGCAGCGGTC GGGCTGAACG GGGGGTTCGT GCACACAGCC CAGCTTGGAG 2040

CGAACGACCT ACACCGAACT GAGATACCTA CAGCGTGAGC TATGAGAAAG CGCCACGCTT 2100

CCCGAAGGGA GAAAGGCGGA CAGGTATCCG GTAAGCGGCA GGGTCGGAAC AGGAGAGCGC 2160

ACGAGGGAGC TTCCAGGGGG AAACGCCTGG TATCTTTATA GTCCTGTCGG GTTTCGCCAC 2220

CTCTGACTTG AGCGTCGATT TTTGTGATGC TCGTCAGGGG GGCGGAGCCT ATGGAAAAAC 2280

GCCAGCAACG CGGCCTTTTT ACGGTTCCTG GCCTTTTGCT GGCCTTTTGC TCACATGTTC 2340

TTTCCTGCGT TATCCCCTGA TTCTGTGGAT AACCGTATTA CCGCCTTTGA GTGAGCTGAT 2400

ACCGCTCGCC GCAGCCGAAC GACCGAGCGC AGCGAGTCAG TGAGCGAGGA AGCGGAAGAG 2460

CGCCTGATGC GGTATTTTCT CCTTACGCAT CTGTGCGGTA TTTCACACCG CATATATGGT 2520

GCACTCTCAG TACAATCTGC TCTGATGCCG CATAGTTAAG CCAGTATACA CTCCGCTATC 2580

GCTACGTGAC TGGGTCATGG CTGCGCCCCG ACACCCGCCA ACACCCGCTG ACGCGCCCTG 2640

ACGGGCTTGT CTGCTCCCGG CATCCGCTTA CAGACAAGCT GTGACCGTCT CCGGGAGCTG 2700

CATGTGTCAG AGGTTTTCAC CGTCATCACC GAAACGCGCG AGGCAGCTGC GGTAAAGCTC 2760
```

(SEQ ID NO:62)

```
ATCAGCGTGG TCGTGAAGCG ATTCACAGAT GTCTGCCTGT TCATCCGCGT CCAGCTCGTT 2820

GAGTTTCTCC AGAAGCGTTA ATGTCTGGCT TCTGATAAAG CGGGCCATGT TAAGGGCGGT 2880

TTTTTCCTGT TTGGTCACTG ATGCCTCCGT GTAAGGGGGA TTTCTGTTCA TGGGGGTAAT 2940

GATACCGATG AAACGAGAGA GGATGCTCAC GATACGGGTT ACTGATGATG AACATGCCCG 3000

GTTACTGGAA CGTTGTGAGG GTAAACAACT GGCGGTATGG ATGCGGCGGG ACCAGAGAAA 3060

AATCACTCAG GGTCAATGCC AGCGCTTCGT TAATACAGAT GTAGGTGTTC CACAGGGTAG 3120

CCAGCAGCAT CCTGCGATGC AGATCCGGAA CATAATGGTG CAGGGCGCTG ACTTCCGCGT 3180

TTCCAGACTT TACGAAACAC GGAAACCGAA GACCATTCAT GTTGTTGCTC AGGTCGCAGA 3240

CGTTTTGCAG CAGCAGTCGC TTCACGTTCG CTCGCGTATC GGTGATTCAT CTGCTAACC 3300

AGTAAGGCAA CCCCGCCAGC CTAGCCGGGT CCTCAACGAC AGGAGCACGA TCATGCGCAC 3360

CCGTGGGGCC GCCATGCCGG CGATAATGGC CTGCTTCTCG CCGAAACGTT TGGTGGCGGG 3420

ACCAGTGACG AAGGCTTGAG CGAGGGCGTG CAAGATTCCG AATACCGCAA GCGACAGGCC 3480

GATCATCGTC GCGCTCCAGC GAAAGCGGTC CTCGCCGAAA ATGACCCAGA GCGCTGCCGG 3540

CACCTGTCCT ACGAGTTGCA TGATAAAGAA GACAGTCATA AGTGCGGCGA CGATAGTCAT 3600

GCCCCGCGCC CACCGGAAGG AGCTGACTGG GTTGAAGGCT CTCAAGGGCA TCGGTCGAGA 3660

TCCCGGTGCC TAATGAGTGA GCTAACTTAC ATTAATTGCG TTGCGCTCAC TGCCCGCTTT 3720

CCAGTCGGGA AACCTGTCGT GCCAGCTGCA TTAATGAATC GGCCAACGCG CGGGGAGAGG 3780

CGGTTTGCGT ATTGGGCGCC AGGGTGGTTT TTCTTTTCAC CAGTGAGACG GCAACAGCT 3840

GATTGCCCTT CACCGCCTGG CCCTGAGAGA GTTGCAGCAA GCGGTCCACG CTGGTTTGCC 3900

CCAGCAGGCG AAAATCCTGT TTGATGGTGG TTAACGGCGG GATATAACAT GAGCTGTCTT 3960

CGGTATCGTC GTATCCCACT ACCGAGATAT CCGCACCAAC GCGCAGCCCG GACTCGGTAA 4020

TGGCGCGCAT TGCGCCCAGC GCCATCTGAT CGTTGGCAAC CAGCATCGCA GTGGGAACGA 4080

TGCCCTCATT CAGCATTTGC ATGGTTTGTT GAAAACCGGA CATGGCACTC CAGTCGCCTT 4140
```

(SEQ ID NO:62)

CCCGTTCCGC TATCGGCTGA ATTTGATTGC GAGTGAGATA TTTATGCCAG CCAGCCAGAC 4200

GCAGACGCGC CGAGACAGAA CTTAATGGGC CCGCTAACAG CGCGATTTGC TGGTGACCCA 4260

ATGCGACCAG ATGCTCCACG CCCAGTCGCG TACCGTCTTC ATGGGAGAAA ATAATACTGT 4320

TGATGGGTGT CTGGTCAGAG ACATCAAGAA ATAACGCCGG AACATTAGTG CAGGCAGCTT 4380

CCACAGCAAT GGCATCCTGG TCATCCAGCG GATAGTTAAT GATCAGCCCA CTGACGCGTT 4440

GCGCGAGAAG ATTGTGCACC GCCGCTTTAC AGGCTTCGAC GCCGCTTCGT TCTACCATCG 4500

ACACCACCAC GCTGGCACCC AGTTGATCGG CGCGAGATTT AATCGCCGCG ACAATTTGCG 4560

ACGGCGCGTG CAGGGCCAGA CTGGAGGTGG CAACGCCAAT CAGCAACGAC TGTTTGCCCG 4620

CCAGTTGTTG TGCCACGCGG TTGGGAATGT AATTCAGCTC CGCCATCGCC GCTTCCACTT 4680

TTTCCCGCGT TTTCGCAGAA ACGTGGCTGG CCTGGTTCAC CACGCGGGAA ACGGTCTGAT 4740

AAGAGACACC GGCATACTCT GCGACATCGT ATAACGTTAC TGGTTTCACA TTCACCACCC 4800

TGAATTGACT CTCTTCCGGG CGCTATCATG CCATACCGCG AAAGGTTTTG CGCCATTCGA 4860

TGGTGTCCGG GATCTCGACG CTCTCCCTTA TGCGACTCCT GCATTAGGAA GCAGCCCAGT 4920

AGTAGGTTGA GGCCGTTGAG CACCGCCGCC GCAAGGAATG GTGCATGCAA GGAGATGGCG 4980

CCCAACAGTC CCCCGGCCAC GGGGCCTGCC ACCATACCCA CGCCGAAACA AGCGCTCATG 5040

AGCCCGAAGT GGCGAGCCCG ATCTTCCCCA TCGGTGATGT CGGCGATATA GGCGCCAGCA 5100

ACCGCACCTG TGGCGCCGGT GATGCCGGCC ACGATGCGTC CGGCGTAGAG GATCGAGATC 5160

GATCTCGATC CCGCGAAATT AATACGACTC ACTATAGGGG AATTGTGAGC GGATAACAAT 5220

TCCCCTCTAG AAGTCGACTT TAAGAAGGAG TACCAAG ATG CCT GAG GAA AGT CAG 5275

GAG ACA TTC GAA GAT CTA TGG AAA CTA CTT CCT GGT CAC CAC CAC CAT 5323

CAC CAT GGT ATG AGC GGC GGC ATG GAG GAG CCC AGT GAC CTT GAG GAG 5371

CTC GAG CAG TTT GCC AAG ACC TTC AAA CAA AGA CGA ATC AAA CTT GGA 5419

TTC ACT CAG GGT GAT GTT GGG CTC GCT ATG GGG AAA CTA TAT GGA AAT 5467

(SEQ ID NO:62)

GAC TTC AGC CAA ACT ACC ATC TCT CGA TTT GAA GCC TTG AAC CTC AGC   5515

TTT AAG AAC ATG TGC AAG TTG AAG CCA CTT TTA GAG AAG TGG CTA AAT   5563

GAT GCA GAG AAC CTC TCA TCT GAT TCG TCC CTC TCC AGC CCA AGT GCC   5611

CTG AAT TCT CCA GGA ATT GAG GGC TTG AGC AGG CGC CGT AAG AAA CGC   5659

ACC AGC ATA GAG ACC AAC ATC CGT GTG GCC TTA GAG AAG AGT TTC TTG   5707

GAG AAT CAA AAG CCT ACC TCG GAA GAG ATC ACT ATG ATT GCT GAT CAG   5755

CTC AAT ATG GAA AAA GAG GTG ATT CGT GTT TGG TTC TGT AAC CGT CGA   5803

CAG AAA GAA AAA AGA ATC AAC CCA CAG CCA GAA CTC GCC CCG GAA GAC   5851

CCC GAG GAT TAGGATCCGA ATT   5873

FIGURE 9

Phosphorylation of Recombinant Substrates by
Purified Human DNA-PK
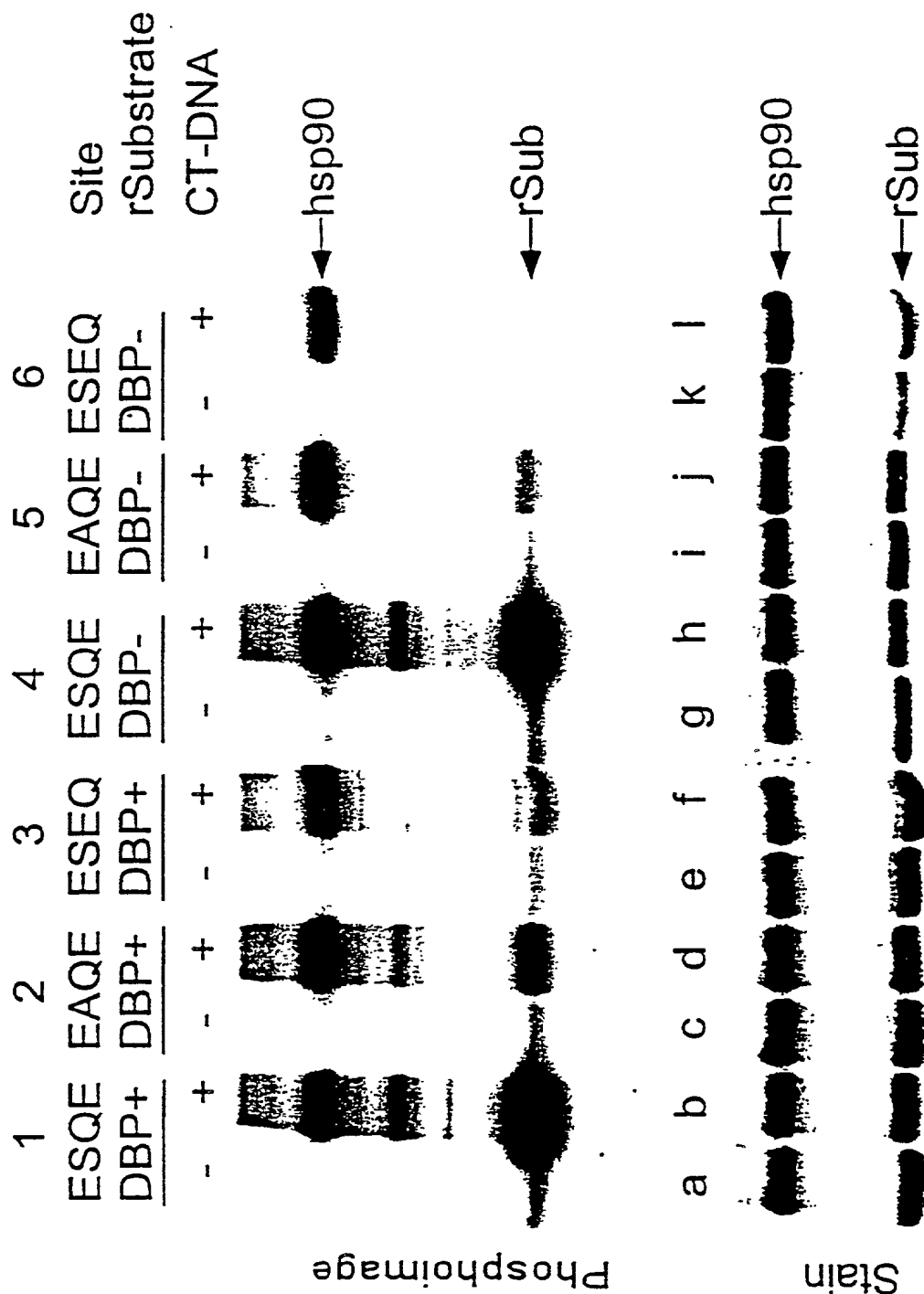
FIGURE 10

Isoelectrofocusing Analysis of Recombinant DNA-PK Substrates

FIGURE 11

p422SUB1
9070 bps

WT-SUB1

MMVT

AmpR

SV40

Neo

SV40

EcoRI, 1
HindIII, 91
Bsu36I, 375

8855, Bsu36I

7270, PstI

6518, EcoRI
6312, PstI
6042, ApaI
5767, BamHI

4882, PstI
4785, Bsu36I
4776, PvuII
4747, SmaI

PvuII
BglII, 3959
PstI, 4014

PvuII, 3746
HindIII, 3654

BamHI, 3426

BamHI, 3079

SacI, 1169
Bsu36I, 1388
PvuII, 1409
NheI, 1544
XbaI, 1550
SalI, 1557
Bsu36I, 1585
BglII, 1611
SacI, 1693
EcoRI, 1938
SalI, 2123
BamHI, 2187
EcoRI, 2193
SacI, 2199
SalI, 2206
HindIII, 2212
NotI, 2218
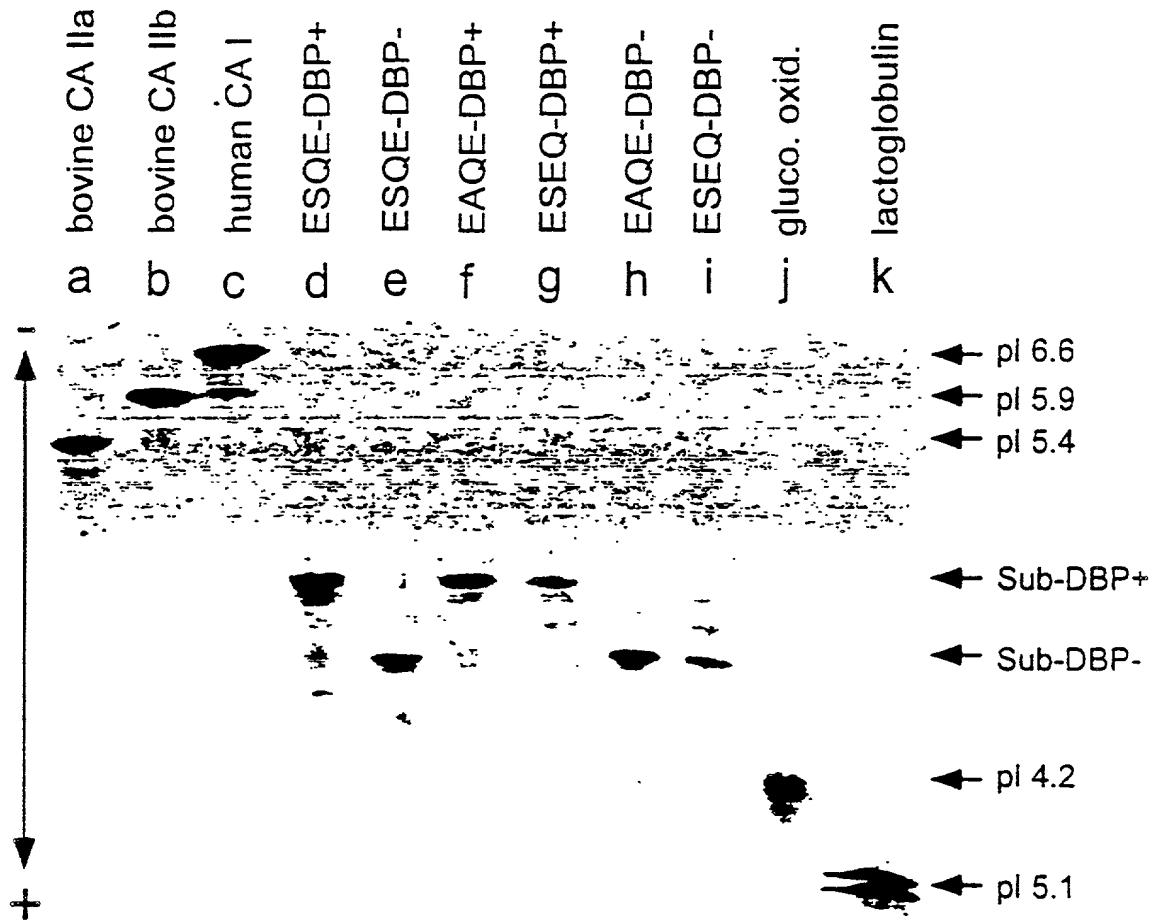
Applicant(s): **Carl W. Anderson, et al.**

For: **DNA-PK Assay**

DECLARATION CLAIMING SMALL ENTITY
STATUS (37 CFR 1.9(f) and 1.27(d) - NONPROFIT ORGANIZATION

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

NAME OF ORGANIZATION ___ Brookhaven Science Associates ___

ADDRESS OF ORGANIZATION ___ Upton, New York 11973 ___

TYPE OF ORGANIZATION

    ___   UNIVERSITY OR OTHER INSTITUTION OF HIGHER EDUCATION

    ___   TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 USC 501 (a) and 501 (c)(3)

    X   NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA

               (NAME OF STATE ___ Delaware ___)
               (CITATION OF STATUTE ___ Title 6, Chapter 18 Delaware Code)

    ___   WOULD QUALIFY AS TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 USC 501 (a) and 501 (c)(3)) IF LOCATED IN THE UNITED STATES OF AMERICA

    ___   WOULD QUALIFY AS NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA IF LOCATED IN THE UNITED STATES OF AMERICA

               (NAME OF STATE _____)
               (CITATION OF STATUTE _____)

I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 CFR 1.9(e) for purposes of paying reduced fees under Section 41(a) and (b) of Title 35, United States Code with regard to the above-identified invention,

described in

   _x_   the specification filed herewith

   ___   application serial no. _____ , filed _____

   ___   patent no. _____, issued _____

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above-identified invention.

If the rights held by the nonprofit organization are not exclusive, each individual, concern or organization having rights to the invention is listed below and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 CFR 1.9(d) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

The U.S. Government, as represented by the U.S. Department of Energy, has a license to the subject invention resulting from a funding agreement pursuant to 35 U.S.C. 202C(4), which license does not preclude the proper claiming of small entity status (M.P.E.P. Section 509.02, "Rights Held by Government Organization").

I acknowledge the duty to file, in this application or patent, notification of any charge in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate [37 CFR 1.28(b)].

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING MARGARET C. BOGOSIAN

TITLE IN ORGANIZATION PATENT COUNSEL

SIGNATURE   DATE

Attorney's Docket No. ___BSA 01-02___ .

COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

TYPE OF DECLARATION

This declaration is of the following type:

- ☒ original
- ☐ design
- ☐ supplemental
- ☐ national stage of PCT
- ☐ divisional
- ☐ continuation
- ☐ continuation-in-part (CIP)

INVENTORSHIP IDENTIFICATION

My residence, post office address and citizenship are as stated below next to my name. I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

TITLE OF INVENTION

"DNA-PK ASSAY"

SPECIFICATION IDENTIFICATION

the specification of which:

(a) ☒ is attached hereto

(b) ☐ was filed on _____ as ☒ Serial No. 07/_____ or ☐ Express Mail No., as Serial No. not yet known _____ and was amended on _____

_____.

(c) ☐ was described and claimed in PCT International Application No. _____ filed on _____ and as amended under PCT Article 19 on _____.

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ACKNOWLEDGEMENT OF REVIEW OF PAPERS AND DUTY OF CANDOR

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information of which I am aware which is material to patentability as defined in 37, Code of Federal Regulations, § 1.56 and which is material to the examination of this application, namely, information where there is a substantial likelihood that a reasonable examiner would consider it important in deciding whether to allow the application to issue as a patent.

PRIORITY CLAIM (35 U.S.C. § 119)

I hereby claim foreign priority benefits under Title 35, United States Code, § 119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed.

(d) ☒   no such applications have been filed.
(e) ☐   such applications have been filed as follows.

A. PRIOR FOREIGN/PCT APPLICATION(S) FILED WITHIN 12 MONTHS (6 MONTHS FOR DESIGN) PRIOR TO THIS APPLICATION AND ANY PRIORITY CLAIMS UNDER 35 U.S.C. § 119

Country (or indicate if PCT)	Application No.	Date of Filing (day, month, year)	Priority Claimed Under 37 USC 119
			☐ Yes ☐ No
			☐ Yes ☐ No
			☐ Yes ☐ No
			☐ Yes ☐ No
			☐ Yes ☐ No

**ALL FOREIGN APPLICATION(S), IF ANY FILED MORE THAN 12 MONTHS
(6 MONTHS FOR DESIGN) PRIOR TO THIS U.S. APPLICATION**

POWER OF ATTORNEY

I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

Name and Registration No.

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Registration No. 25,324

Christine L. Brakel
Registration No. 45,772

Lori-Anne Mooney
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DIRECT TELEPHONE CALLS TO:
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    (631) 344-7338

DECLARATION

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

SIGNATURE(S)

Full name of sole or first inventor

<u>Carl</u>   <u>W.</u>   <u>Anderson</u>
(Given Name)   (Middle Initial or Name) Family (or last name)

Inventor's signature_____

Date_____ Country of Citizenship <u>USA</u>

Residence___ <u>Stony Brook, New York</u>

Post Office Address <u>23 Shelbourne Lane</u>

_____ <u>Stony Brook, New York 11790</u>

Full name of second joint inventor, if any

<u>Margery</u>   <u>A.</u>   <u>Connelly</u>
(Given Name)   (Middle Initial or Name) Family (or last name)

Inventor's signature_____

Date_____ Country of Citizenship <u>USA</u>

Residence___ <u>Medford, New York</u>

Post Office Address___ <u>215 Mt. Vernon Ave</u>

_____ <u>Medford, New York 11763</u>

CHECK PROPER BOX(ES) FOR ANY OF THE FOLLOWING ADDED PAGE(S) WHICH
FORM A PART OF THIS DECLARATION

  ☐ Signature by administrator(trix), executor(trix) or legal
     representative for deceased or incapacitated inventor.
     Number of pages added _____

          * * *

  ☐ Signature for inventor who refuses to sign or cannot be
     reached by person authorized under 37 CFR 1.47. Number
     of pages added _____

          * * *

  ☐ Added page for signature by one joint inventor on behalf
     of deceased inventor(s) where legal representative cannot
     be appointed in time (37 CFR 1.47).

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* * *

☒　Added pages to combined declaration and power of attorney for divisional, continuation or continuation-in-part (C-I-P) application.

☒ Number of pages added ___1___

* * *

☐　Authorization of attorney(s) to accept and follow instructions from representative.

* * *

(If no further pages form a part of this Declaration, then end this Declaration with this page and check the following item:)

☐　This declaration ends with this page.

ADDED PAGE TO COMBINED DECLARATION AND POWER OF
ATTORNEY FOR DIVISIONAL, CONTINUATION OR CIP APPLICATION

CLAIM FOR BENEFIT OF EARLIER U.S. APPLICATION(S) UNDER 35 U.S.C. 120

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s) that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose information of which I am aware that is material to the examination of this application, namely, information where there is substantial likelihood that a reasonable Examiner would consider it important in deciding whether to allow the application to issue as a patent, which occurred between the filing date of the prior application(s) and the filing date of this application.

PRIOR U.S. APPLICATIONS FOR BENEFIT UNDER 35 USC 120:				
U.S. APPLICATIONS		STATUS		
U.S. Applications	U.S. Filing Date	Patented	Pending	Abandoned
08/132,284	10/6/93		X	

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